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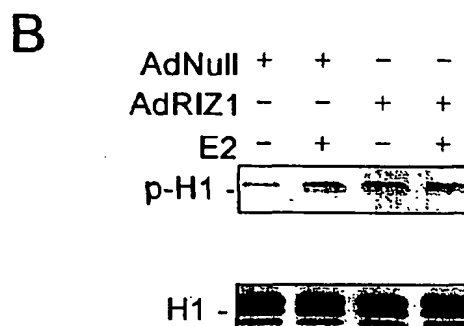
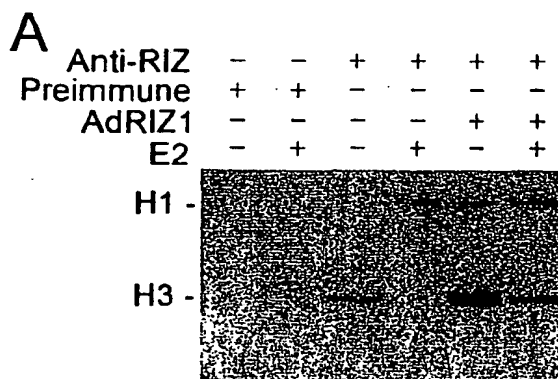
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(54) Title: SCREENING, DIAGNOSTIC AND THERAPEUTIC METHODS RELATING TO RIZ



(57) Abstract: The invention provides a method of screening for a compound that modulates RIZ histone methyltransferase (HMT) activity, by contacting a RIZ or RIZ fragment having HMT activity with one or more candidate compounds, and determining histone methyltransferase activity of the contacted RIZ or RIZ fragment. Also provided is a method of screening for a compound that modulates progesterone receptor (PR) activity, by providing a RIZ1 modulatory compound, and determining the ability of the RIZ1 modulatory compound to modulate PR activity. Further provided is a method of identifying an individual with an estrogen receptor positive (ER+) tumor having a reduced likelihood of responding to endocrine therapy. The method involves determining the RIZ1 status of the tumor, wherein an abnormal RIZ1 status identifies the individual as an individual with a reduced likelihood of responding to endocrine therapy.

WO 02/092002, A2

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SCREENING, DIAGNOSTIC AND THERAPEUTIC  
METHODS RELATING TO RIZ

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The U.S. Government has certain rights in this invention.

BACKGROUND OF THE INVENTION

FIELD OF THE INVENTION

This invention relates generally to the fields  
10 of cancer biology and endocrinology and, more  
specifically, to biological activities of RIZ  
polypeptides.

BACKGROUND INFORMATION

Post-translational addition of methyl groups to  
15 the amino-terminal tails of histone proteins is catalyzed  
by a family of proteins known as histone  
methyltransferases (HMTs). Histone site-specific  
methylation is associated with a variety of fundamental  
cellular processes, including transcriptional regulation,  
20 epigenetic silencing and heterochromatin formation. Loss  
of HMT function is expected to directly contribute to the  
de-differentiation and genomic instability that are  
characteristic of cancer.

Further implicating HMTs in cancer, it has been shown that a deficiency in dietary methionine or folate causes cancer. Dietary methionine and folate in turn regulate the cellular levels of S-adenosylmethionine (SAM), which is used as a methyl group donor by methyltransferases. Additionally, several independent lines of investigation have revealed that alterations in the methionine metabolic pathway, which can lead to a deficiency in SAM and/or an increase in the methyltransferase inhibitor S-adenosylhomocysteine (SAH), are associated with cancer.

Thus, there exists a need to identify proteins with histone methyltransferase activity, determine their substrates and regulators, and identify compounds that modulate their activity. In particular, there exists a need to identify histone methyltransferase activity within proteins already recognized to play critical roles in regulating cell proliferation. Compounds that modulate the HMT activity of such proteins are expected to be useful as therapeutics to regulate cell growth.

The steroid hormone progesterone is a critical component of the female reproductive cycle and is required to maintain pregnancy. The molecular target of progesterone is the intracellular progesterone receptor. Upon binding to progesterone, the progesterone receptor translocates to the nucleus where it binds as a transcription factor to DNA transcriptional elements present in progesterone-regulated genes.

Ligands to the progesterone receptor play an important role in female reproductive medicine and cancers. For example, progesterone and its synthetic analogs are useful in birth control formulations, in treating endometriosis and in maintaining pregnancy.

Antagonists to progesterone are useful in treating chronic disorders such as certain forms of hormone dependent cancer of the breast, ovaries and endometrium (the lining of the uterus) and in treating uterine  
5 fibroids. Progesterone antagonists are also useful in combination with other drugs for terminanting early stage pregnancies.

Compounds that modulate progesterone receptor activity are thus expected to be useful in reproductive  
10 applications and for treating malignant disorders. However, suitable high-throughput assays for identifying such compounds are currently lacking, in part due to a lack of understanding of physiologically relevant interactions of the progesterone receptor with cellular  
15 molecules. Thus, there exists a need for improved methods to screen for compounds that modulate progesterone receptor activity.

The steroid hormone estrogen directly and indirectly promotes the proliferation of tissues  
20 expressing estrogen receptors. Endocrine therapy to reduce the proliferative effects of estrogens, including therapy with selective estrogen receptor modulators (SERMs) such as tamoxifen, is currently the preferred first-line therapy in patients with estrogen receptor  
25 positive (ER+) tumors. However, a significant portion of patients with ER+ tumors do not respond to endocrine therapy. In these patients, alternative therapies are warranted in order to improve the odds of survival. Currently, there is no satisfactory method of predicting  
30 which ER+ tumors will respond to endocrine therapy.

Thus, there exists a need to identify molecules that correlate with loss of estrogen responsiveness in ER+ tumors, in order to more accurately determine which

individuals are more or less likely to respond to endocrine therapy. Using such a correlation, an individualized course of treatment with an improved likelihood of success can be chosen.

- 5                   The present invention satisfies these needs, and provides related advantages as well.

#### SUMMARY OF THE INVENTION

The invention provides a method of screening for a compound that modulates RIZ histone  
10 methyltransferase activity. The method involves contacting a RIZ or RIZ fragment having histone methyltransferase activity with one or more candidate compounds, and determining histone methyltransferase activity of the contacted RIZ or RIZ fragment. A  
15 compound that modulates RIZ histone methyltransferase activity is identified.

The invention also provides a method of screening for a compound that modulates progesterone receptor activity. The method involves providing a RIZ1  
20 modulatory compound, and determining the ability of the RIZ1 modulatory compound to modulate progesterone receptor activity. A compound that modulates progesterone receptor activity is thereby identified.

Also provided is a method of identifying an  
25 individual with an estrogen receptor positive (ER+) tumor having a reduced likelihood of responding to endocrine therapy. The method involves determining the RIZ1 status of the tumor, wherein an abnormal RIZ1 status identifies the individual as an individual with a reduced likelihood  
30 of responding to endocrine therapy.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1A shows the effect of RIZ1 on hormone responsive promoters. The indicated amounts of RIZ1 and various nuclear hormone receptor expression vectors were  
5 cotransfected with a reporter construct containing the appropriate hormone receptor response element into CV-1 cells. Cells were treated with or without the indicated hormone for 24 h and assayed for CAT activity. Data shown represent the means  $\pm$  SEM of three independent  
10 experiments.

Figure 1B shows that RIZ1 forms hormone-dependent protein complexes *in vivo* with ER $\alpha$  and progesterone receptor proteins. T47-D (for progesterone receptor) and MCF-7 (for ER) cells were infected with  
15 adenovirus expressing RIZ1. Cell extracts treated with or without hormones were prepared and immunoprecipitated (IP) with preimmune serum or monoclonal antibodies against progesterone receptor (PR) (detecting both PR-B and PR-A) or ER $\alpha$ , as indicated. The immunoprecipitated  
20 products were analyzed by immunoblot analysis using RIZ1, progesterone receptor or ER $\alpha$  monoclonal antibodies (2D7, 1A6, and sc-8005, respectively).

Figure 1C shows immunohistochemistry analysis of RIZ expression in target organs of female sex steroid  
25 hormones. Tissues from 7-week old virgin wild-type mice were analyzed for expression of RIZ and ER $\alpha$ . The sections show RIZ and ER $\alpha$  immunostaining in uterus (panels a and b), breast (c and d), and vaginal epithelium (e and f). Scale bar in a, c, and e also  
30 applies to b, d, and f respectively.

Figure 1D shows RT-PCR analysis of RIZ1 expression in target organs of female sex steroid

hormones. Total RNAs isolated from the indicated tissues of mice were used for RT-PCR analysis.

Figure 2 shows uterine and vaginal responses to female sex steroid hormones in RIZ1 mutant mice. A and B, Uterine wet weight was measured and the ratio of uterine weight to body weight calculated for the indicated number of mice and presented as mean  $\pm$  SEM. C. The uterine response to a decidual response was measured. After treatment with E2 and progesterone, and mechanical stimulation of the left uterine horn, the ratio of the weights of the stimulated (L) to the unstimulated (control; R) horn was calculated for the indicated number of mice and presented as mean  $\pm$  SEM. D and E. Vaginal responses. Vaginal tissues were collected and fixed for H & E staining. The thickness of the entire vaginal epithelium and the cornified layer were measured at 7 randomly selected areas at the maximum depth of the vaginal epithelium in each mouse and data are presented as mean  $\pm$  SEM for 7 mice in each group. F and G. Impaired regulation of uterine progesterone receptor expression by E2 in RIZ1 mutant mice. Uteri were collected and immunohistochemically analyzed for progesterone receptor expression. Estrogen treated uteri are shown in G (top panel: low magnification; bottom panel: high magnification). Randomly selected areas were photographed and more than 1000 cells from each compartment of the uteri of 4 mice in each group were counted, and data is presented as mean  $\pm$  SEM (G). Inserted scale bars apply for both genotypes.

Figure 3 shows mammapoiesis in RIZ1 deficient mice. Whole mounts of the fourth mammary gland of mice with the indicated genotypes were prepared and stained. A and B, Seven week old virgin mice. C and D, Mice pregnant for the first time. E and F, Higher

magnification of the ducts and alveolar structures of the mammary glands of the pregnant mice. G and H, Mammary glands from mice treated with hormone pellets containing P and E2 as described in methods. I and J, Higher magnification of the mammary ducts and alveolar structures from G and H, respectively. Scale bars that apply to both genotypes are inserted.

Figure 4 shows modulation by RIZ of E2-dependent transcription mediated by endogenous ER. MCF-7 cells (A) and CV-1 cells (B) were cotransfected with the reporter ERE-tk-CAT and either RIZ1 or RIZ2 expression plasmids. Cells were treated with or without E2 and assayed for CAT activity. Data shown represent the means  $\pm$  SEM of three independent experiments.

Figure 5 shows methylation of histones in vitro by the PR-domain, the PR-binding domain (PBD), and the combined action of the two domains. Bacteria-expressed GST fusion proteins of human RIZ1 were used for in vitro HMT reactions using histones as substrates. Top panel shows Coomassie blue staining of purified proteins and histones. Bottom panel shows fluorography indicating methylation of substrates. A. Methylation of H4 by GST-RIZ200 (consisting of RIZ1 residues 1-200) but not by GST protein alone, GST-RIZ161 (consisting of RIZ1 residues 1-161), and three GST-RIZ200 point mutant proteins as indicated. B. Methylation of H4 by GST-RIZ332 (consisting of RIZ1 residues 1-332). C. Enhanced methylation conferred by GST-RIZ322 plus GST-RIZPBD.

Figure 6 shows methylation of histones by full length RIZ1 protein expressed in mammalian cells. A. Nuclear extracts (500  $\mu$ g) prepared from U20S cells infected with AdRIZ1 virus or AdNull control virus were



immunoprecipitated with preimmune serum or RIZ1 serum anti-KG7.1S. Immunoprecipitates were then assayed for HMT activity using free histones as substrates. The panel shows fluorography indicating methylation of histone H1, H3 and H4. B. Enhanced methylation of lysine 9 on H3 by RIZ1 overexpression. Histones were extracted from U20S cells infected with AdRIZ1 or control virus and analyzed by western blot analysis using either antibody for methylated K9 on H3 or methylated K4 on H3. C. Methylation of H3 N terminus peptide in vitro by RIZ1. In vitro methylation assays using immunoprecipitated RIZ1 as enzyme and the indicated N-terminal 1-20 residue peptide of H3 and acetylated H3 (K9/K14 Ac: acetylated on K9 and K14 residues) as substrates. The methylated peptides were resolved by 16% Tris-Tricine SDS gel followed by fluorography. D. Automated sequencing of the H3 N-terminal peptide (residues 1-20) that had been methylated in vitro by full length RIZ1 purified by immunoprecipitation. The tritium incorporation of individual amino acids identified at each successive round of microsequencing is shown.

Figure 7 shows regulation of RIZ1 methyltransferase activity by estrogen. A. Effects of estrogen on RIZ1 in MCF7 cells. Same amounts of nuclear extracts (500 µg) from E2 treated or control MCF-7 cells, or from AdRIZ1 infected cells were immunoprecipitated with preimmune serum or RIZ1 serum anti-KG7.1S. Immunoprecipitates were then assayed for HMT activity using free histones as substrates. The panel shows fluorography indicating methylation of histone H1 and H3. B. Effects of E2 and RIZ1 on H1 phosphorylation. MCF7 cells infected with control or AdRIZ1 virus were treated with E2 or vehicle for 48 hr. Histones were extracted after treatment and analyzed by western blot using

anti-phosph-H1 (top panel) or anti-H1 antibodies (bottom panel).

#### DETAILED DESCRIPTION OF THE INVENTION

The retinoblatoma-protein-interacting zinc  
5 finger gene, or "RIZ" gene, was originally isolated in a  
functional screen for proteins that bind to the Rb tumor  
suppressor (Buyse et al., Proc. Natl. Acad. Sci. USA  
92:4467-4471 (1995)). Two products of the RIZ gene are  
produced, owing to alternative promoter usage. RIZ2 is  
10 identical to RIZ1 except that it lacks the N-terminal 200  
(rat) or 201 (human) amino acids (Liu et al., J. Biol.  
Chem. 272:2984-2991 (1997)).

RIZ1 is considered to be a tumor suppressor  
gene, as indicated by the following lines of evidence.  
15 The RIZ gene maps to chromosome 1p36, one of the most  
commonly deleted regions in human cancers. Gene  
silencing of RIZ1, but not RIZ2, is common in many types  
of human tumors - including breast cancer, liver cancer,  
colon cancer, neuroblastoma, melanoma, lung cancer and  
20 osteosarcoma (Chadwick et al., Proc. Natl. Acad. Sci. USA  
97:2662-2667 (2000); He et al., Cancer Res. 58:4238-4244  
(1998); Jiang et al., Int. J. Cancer 83:541-547 (1999)) -  
and is associated with promoter DNA methylation (Du et  
al., Cancer Res. 22:8094-8099 (2001)). RIZ1 missense  
25 mutations (Steele-Perkins et al., Genes Dev. 15:2250-2262  
(2001)) and frameshift mutations (Chadwick et al., supra  
(2000); Piao et al., Cancer Res. 60:4701-47-4 (2000);  
Sakurada et al., Genes, Chroms. Cancer 30:207-211 (2001))  
are common in human tumors. RIZ1 expression induces  
30 cell-cycle arrest, apoptosis induction and suppression of  
xenograft tumors (Chadwick et al., supra 2000; He et al.,  
supra (1998); Jiang et al., supra (1999); Jiang and  
Huang, Cancer Res. 61:1796-1798 (2001)). Finally, mouse

gene knock-out models show that inactivation of RIZ1, while retaining normal RIZ2, causes tumor susceptibility (Steele-Perkins et al., supra (2001)). These studies suggest an important function for the N-terminus of RIZ1  
5 in its tumor suppressor activity.

The N-terminus of RIZ1 contains a domain of about 130 residues called the "PR-domain," between about residues 30-160 of RIZ1. The PR-domain was initially identified as a region that showed homology between RIZ1  
10 and the transcriptional repressor PRDI-BF1. This domain was later found to be structurally related to the SET-domain (Huang et al., J. Biol. Chem. 273:15933-15940 (1988)), which was recently shown to be a catalytic motif of lysine histone methyltransferases (HMTs) (Rea et al.,  
15 Nature 406:593-599 (2000)). Despite the sequence similarity between PR and SET, the PR and SET domains are clearly distinct. First, identities among PR-domains or among SET domains are usually about 40%, whereas identities between PR and SET domains are typically only  
20 about 20-30%. Second, SET domains are primarily found at the carboxyl termini of proteins, whereas PR-domains are mostly located at the amino termini. Prior to the instant disclosure, no enzymatic activity of a PR-domain had been described. Furthermore, histone  
25 methyltransferase activity of the PR-domain of RIZ1, or of other domains within RIZ, had not been described.

As disclosed herein, both RIZ1 and RIZ2 proteins have histone methyltransferase activity.  
30 Despite its actions as a tumor suppression, RIZ1 also acts as an estrogen receptor (ER) and progesterone receptor coactivator involved in regulating the normal proliferation and differentiation of estrogen- and progesterone-responsive tissues (see Example I). Because  
35 of the dual role of RIZ as a growth suppressor and growth

promoter, compounds that modulate RIZ histone methyltransferase activity are expected to be useful in applications in which it desirable to positively or negatively modulate cell growth, including treatment of hyperproliferative disorders (e.g. neoplasia, hyperplasia, inflammatory conditions and the like) and treatment of hypoproliferative disorders (e.g. various disorders of hematopoiesis, wound healing and the like).

The invention thus provides a method of screening for a compound that modulates RIZ histone methyltransferase activity. The method is practiced by contacting a RIZ or RIZ fragment having histone methyltransferase activity with one or more candidate compounds, and assaying histone methyltransferase activity of the contacted RIZ or RIZ fragment. A compound that modulates histone methyltransferase activity of the RIZ or RIZ fragment is thereby identified.

As disclosed herein, HMT activity is present both in the PR-domain specific to RIZ1, and also in the C-terminal PR-binding domain (or PBD) common to RIZ1 and RIZ2. Accordingly, either RIZ1 or RIZ2, or fragments thereof that retain HMT activity, can be used in the invention methods.

The RIZ gene from several species has been cloned and characterized (Buyse et al., Proc. Natl. Acad. Sci. USA 92:4467-4471 (1995); Liu et al., supra (1997)). Accordingly, a RIZ polypeptide from any species can be used in the invention screening methods. The human RIZ gene encodes the RIZ1 amino acid sequence set forth as SEQ ID NO:1 (GenBank Accession No. AAC50820; gi:9955379) and the RIZ2 amino acid sequence set forth as SEQ ID NO:2. The rat RIZ gene encodes the RIZ1 amino acid

sequence set forth in SEQ ID NO:3 (GenBank Accession No. AAA74468; gi:949996) and the RIZ2 amino acid sequence set forth as SEQ ID NO:4. The mouse RIZ gene has been partially sequenced (U.S. Patent No. 5,811,304).

5

Human and rat RIZ1 and RIZ2 polypeptides are highly homologous over their entire lengths (see, for example, U.S. Patent Nos. 6,323,335; 6,069,231; and 5,811,304). Because of this high degree of identity across two mammalian species, it is expected that other naturally occurring mammalian RIZ polypeptides, such as RIZ polypeptides from non-human primates, mouse, rabbit, bovine, porcine, ovine, canine or feline species, as well as naturally occurring RIZ polypeptides from other vertebrates, including fish, birds, reptiles and amphibians (e.g. *Xenopus*) will also exhibit a high degree of identity across their lengths with human and rat RIZ.

Using knowledge of the human or rat or RIZ-encoding nucleic acid sequences and polypeptides, those skilled in the art can readily clone RIZ-encoding genes from other species using conventional cDNA or expression library screening methods, or using the polymerase chain reaction (PCR). Additionally, using knowledge of the human or rat RIZ-encoding nucleic acid sequences and polypeptides, those skilled in the art can readily determine cDNA and coding sequences from other species from an analysis of ESTs and genomic sequences present in available databases. From these sequences, full-length RIZ1 or RIZ2 polypeptides, and fragments thereof with HMT activity, can be prepared for use in the screening methods disclosed herein.

A RIZ polypeptide useful in the methods of the invention can also have one or more minor modifications to the naturally occurring sequence, such as one or more

substitutions, additions or deletions. A modified RIZ polypeptide will generally retain at least 70%, 75%, 80%, 90%, 95%, 98% or higher identity with a native RIZ sequence. Such modifications can be advantageous, for example, in enhancing the stability, bioavailability, bioactivity or immunogenicity of the polypeptide, or to facilitate its purification.

Substitutions to a RIZ amino acid sequence can either be conservative or non-conservative. Conservative amino acid substitutions include, but are not limited to, substitution of an apolar amino acid with another apolar amino acid (such as replacement of leucine with an isoleucine, valine, alanine, proline, tryptophan, phenylalanine or methionine); substitution of a charged amino acid with a similarly charged amino acid (such as replacement of a glutamic acid with an aspartic acid, or replacement of an arginine with a lysine or histidine); substitution of an uncharged polar amino acid with another uncharged polar amino acid (such as replacement of a serine with a glycine, threonine, tyrosine, cysteine, asparagine or glutamine); or substitution of a residue with a different functional group with a residue of similar size and shape (such as replacement of a serine with an alanine; an arginine with a methionine; or a tyrosine with a phenylalanine).

Additions to a RIZ amino acid sequence include, but are not limited to, the addition of "tag" sequences, which are conveniently added at the N- or C- termini, after the signal peptide, or within extracellular or intracellular loops. Such tag sequence include, for example, epitope tags, histidine tags, glutathione-S-transferase (GST), fluorescent proteins (e.g. Enhanced Green Fluorescent Protein (EGFP)) and the like. Such additional sequences can be used, for example, to

facilitate expression, purification or characterization of the RIZ polypeptide.

Deletions to a RIZ amino acid sequence include, but are not limited to, deletion of residues at the N- or C- termini that are not critical for function. Deleted sequences can optionally be replaced by tag sequences or fusion sequences, as described previously.

Modifications to a RIZ amino acid sequence can be randomly generated, such as by random insertions, deletions or substitutions of nucleotides in a nucleic acid molecule encoding the polypeptide. Alternatively, modifications can be directed, such as by site-directed mutagenesis of a nucleic acid molecule encoding the polypeptide.

Guidance in modifying the sequence of a RIZ polypeptide while retaining biological activity can be provided by the alignment of the sequence of the RIZ orthologs from human and rat. It is well known in the art that evolutionarily conserved amino acid residues are more likely to be important for maintaining biological activity than less well-conserved residues. Thus, it would be expected that substituting a residue that is highly conserved among RIZ polypeptides across species with a non-conserved residue may be deleterious, whereas making the same substitution at a residue which varies among species would likely not have a significant effect on biological activity. An alignment of the rat and human RIZ1 amino acid sequences is presented in Figure 10 of U.S. Patent No. 5,811,304.

Computer programs well known in the art can also provide guidance in predicting which amino acid

residues can be modified without abolishing a structural or functional feature of a RIZ polypeptide.

As disclosed herein, RIZ histone methyltransferase activity is present in an N-terminal, PR-domain containing portion specific to RIZ1. Accordingly, an exemplary RIZ or RIZ fragment useful in the methods of the invention contains amino acids 1-200 of RIZ1, such as amino acids 1-332 of RIZ1. A further exemplary RIZ or RIZ fragment useful in the methods of the invention contains amino acids 1-520 of RIZ1. In contrast, a RIZ fragment consisting of residues 1-161 of human RIZ1 lacked HMT activity, as did a RIZ1 containing missense mutations in the PR-domain found in tumors, namely C106Y, I88V and A159V.

Additionally, as disclosed herein, RIZ histone methyltransferase activity is also present in a C-terminal PR-binding domain (PBD) containing portion common to RIZ1 and RIZ2 (Huang et al., J. Biol. Chem. 273:15933-15940 (1988)). Accordingly, an exemplary RIZ or RIZ fragment useful in the methods of the invention contains amino acids 1514-1680 of RIZ1, such as amino acids 1514 to the C-terminus of RIZ1.

The HMT activity of other RIZ polypeptides and fragments, including polypeptides and fragment with modified sequences as described above, can readily be determined by the assays described herein in order to select a RIZ or RIZ fragment having histone methyltransferase activity for use in the screening methods.

As used herein, the term "histone methyltransferase activity" or "HMT activity," with respect to a RIZ or RIZ fragment, refers to the ability



of the RIZ or RIZ fragment to catalyze the methylation of histones or histone peptides under suitable assay conditions. In contrast, under the same conditions, a control polypeptide, such as glutathione-S-transferase (GST), will not be able to catalyze the methylation of histones or histone peptides. HMT activity can be exhibited either in an *in vitro* assay with purified or partially purified RIZ, or in a cell-based assay. HMT activity includes HMT activity exhibited toward any histone, such as histone H1, H2A, H2B, H3 or H4.

Histones suitable for use as substrates in HMT activity assays can be obtained commercially (e.g. from Roche Molecular Biochemicals), prepared recombinantly based on known nucleic acid sequences, or extracted from cells using methods known in the art. Histone peptides suitable for use as substrates in HMT assays, including peptides with native sequences and peptides modified by acetylation on lysine residues, can be obtained commercially (e.g. from Upstate Biotech) or produced synthetically. Suitable histone peptides include, for example, H3 N-terminal peptides that include lys-9 (K9) (e.g. H3 amino acids 1-20) and H1 N-terminal peptides that include lys-25 (K25) (e.g. H1 amino acids 15-37 or 12-31). The methylation site on H4 is expected to be lys-20 (K20). Accordingly, a suitable histone H4 peptide can be an N-terminal peptide that includes lys-20.

For HMT assays, a RIZ or RIZ fragment of any desired sequence can conveniently be produced recombinantly, such as by expression of the encoding nucleic acid molecule in bacteria, yeast, insect or mammalian cells. The expressed polypeptide can then be isolated with anti-RIZ antibodies, or purified or partially purified by standard biochemical fractionation methods. Alternatively, to facilitate isolation, the RIZ

- polypeptide can be expressed as a fusion with a tag sequence, such as glutathione-S-transferase (GST), a 6XHis tag or an epitope tag. Methods of producing and isolating tagged and untagged recombinant proteins are well known in the art (see, for example, Sambrook et al., Molecular Cloning: A Laboratory Manual, 3rd ed., Cold Spring Harbor Press, Plainview, New York (2001); Ausubel et al. (Current Protocols in Molecular Biology (Supplement 47), John Wiley & Sons, New York (1999)).
- 10 As an alternative to preparing a recombinant RIZ or RIZ fragment, an endogenous RIZ1 or RIZ2 can be purified or partially purified from a convenient cell or tissue source. A RIZ fragment can thus be prepared by enzymatic or chemical cleavage of the endogenous RIZ.
- 15 Alternatively, a RIZ fragment can be prepared by synthetic methods.
- HMT activity of a RIZ can be determined by methods known in the art. For example, the RIZ and a histone or histone peptide can be incubated with a
- 20 labeled methyl donor, such as S-adenosyl-[methyl-<sup>14</sup>C]-L-methionine, or S-adenosyl-[methyl-<sup>3</sup>H]-L-methionine, under suitable assay conditions. Transfer of the radiolabel to the histone or histone peptide can be detected, for example, by SDS-PAGE
- 25 electrophoresis and fluorography. Alternatively, following the reaction the histone or histone peptides can be separated from the methyl donor by filtration, and the amount of radiolabel retained on the filter quantitated by scintillation counting. Other suitable
- 30 labels that can be attached to methyl donors, such as chromogenic and fluorescent labels, and methods of detecting transfer of these labels to histones and histone peptides, are known in the art.

Alternatively, HMT activity of a RIZ can be determined using an unlabeled methyl donor (e.g. S-adenosyl-L-methionine) and reagents that selectively recognize methylated histones or histone peptides. For example, after incubation of the RIZ, methyl donor and histones or histone peptides, under suitable assay conditions, methylated histones or histone peptides can be detected by immunoblotting or by an ELISA assay with antibodies specific for methylated histone epitopes. Suitable antibodies are described, for example, in Nakayama et al., Science 292:110-113 (2001), Noma et al., Science 293:1150-1155 (2001) and published U.S. Patent Application No. 20020039776, or can be prepared by methods known in the art (see Harlow and Lane, Antibodies: A Laboratory Manual, Cold Spring Harbor Laboratory Press (1988)).

Instead of using antibodies, methylated histones can be detected using reagents that selectively bind methylated histones with high affinity. Such reagents are known in the art or can be determined by screening assays known in the art. An exemplary binding reagent is heterochromatin protein HP1, which binds histone H3 when methylated at lysine 9 (H3-K9). HP1, or a binding fragment thereof, can be labeled, and the HP1 or fragment bound to methylated H3-K9 detected. Alternatively, the HP1 or fragment need not be labeled, and can instead be detected using an anti-HP1 antibody in an ELISA assay.

Various low-throughput and high-throughput enzyme assay formats are known in the art and can be readily adapted for RIZ HMT assays. For high-throughput assays, the histone or histone peptide substrate can conveniently be immobilized on a solid support, such as a multiwell plate, slide or chip. Following the reaction,

the methylated product can be detected on the solid support by the methods described above. Alternatively, the HMT reaction can take place in solution, after which the histone or histone peptide can be immobilized on a solid support, and the methylated product detected. To facilitate such assays, the solid support can be coated with streptavidin and the histone labeled with biotin, or the solid support can be coated with anti-histone antibodies. The skilled person can determine suitable assay formats depending on the desired throughput capacity of the screen.

The invention screening method involves contacting a RIZ or RIZ fragment having histone methyltransferase activity with one or more candidate compounds and assaying histone methyltransferase activity of the contacted RIZ or RIZ fragment. A candidate compound useful in the methods of the invention can be a naturally occurring macromolecule, such as a peptide, nucleic acid, carbohydrate, lipid, or any combination thereof. A candidate compound alternatively can be a partially or completely synthetic derivative, analog or mimetic of such a macromolecule, or a small, synthetic molecule, such as an organic molecule prepared by combinatorial chemistry methods. A candidate compound can be detectably labeled or attached to a solid support, if desired, as appropriate in a particular assay.

Methods for producing large libraries of compounds, including simple or complex organic molecules, metal-containing compounds, carbohydrates, peptides, proteins, peptidomimetics, glycoproteins, lipoproteins, nucleic acids, antibodies, and the like, are well known in the art and are described, for example, in Huse, U.S. Patent No. 5,264,563; Francis et al., Curr. Opin. Chem. Biol. 2:422-428 (1998); Tietze et al., Curr. Biol.,

2:363-371 (1998); Sofia, Mol. Divers. 3:75-94 (1998);  
Eichler et al., Med. Res. Rev. 15:481-496 (1995).  
Libraries containing large numbers of natural and  
synthetic compounds also can be obtained from commercial  
5 sources.

The number of different candidate compounds to  
screen in a particular assay can be determined by those  
skilled in the art, and can be 2 or more, such as 5, 10,  
15, 20, 50 or 100 or more different compounds. For  
10 certain applications, such as when a library of random  
compounds is to be screened, and for automated  
procedures, it may be desirable to screen  $10^3$  or more  
compounds, such as  $10^5$  or more compounds, including  $10^7$  or  
more compounds. If desired, a plurality of candidate  
15 compounds can be assayed in a pool, and the pool  
repeatedly subdivided until a single compound with the  
desired activity is identified. Candidate compounds can  
be assayed simultaneously, in parallel, or sequentially.

The amount of candidate compound to use in a  
20 reaction can be determined by the skilled person based on  
the nature of the compound, the nature of the assay, and  
the concentration of the reactants. If desired, a range  
of doses of candidate compound can be tested.

Generally, the candidate compound will be  
25 included in an HMT reaction together with the RIZ,  
histone or histone peptide substrate, and methyl donor.  
Optionally, the candidate compound and the RIZ can first  
be incubated together, and then the other reactants  
added. If desired, other components, such as different  
30 RIZ polypeptides or RIZ fragments, can be included in the  
reactions, and the effect of the candidate compound on  
modulating RIZ HMT activity under such conditions

determined. The skilled person can determine suitable combinations of reactants and components.

For cell-based screening assays, a cell expressing a RIZ can be contacted with a candidate compound. Either the *in vivo* methylation of isolated histones can be determined following contacting, or the RIZ polypeptide can be isolated and its activity in methylating isolated histones or histone fragments assayed as described above. If desired, the ability of a candidate compound to modulate RIZ activity under physiologically relevant conditions can be determined in cell-based screening assays. For example, the ability of a candidate compound to modulate RIZ activity in the presence of estrogen or progesterone, in response to co-expressed polypeptides, or in tumor cells, can be determined.

Determining whether a candidate compound modulates RIZ HMT activity, either positively or negatively, generally requires comparison to a control. A control can be an identical reaction to the test reaction, except the control is not exposed to the candidate compound. The HMT activity of the control reaction can be assessed either before, after, or at the same time as the test reaction. A compound that "modulates" HMT activity is a compound that increases or decreases HMT activity, in comparison to a control, by at least 2-fold, such as at least 5-fold, 10-fold or more.

The results described in Example I suggest that the H3-K9 methylation activity of RIZ1 is linked with growth arrest, whereas its H1-K25 methylation activity is associated with cell proliferation. Accordingly, compounds that differentially regulate these two activities can be identified and used to promote or

inhibit cell growth, as appropriate for a particular application.

As disclosed herein, RIZ1, but not RIZ2, acts as a transcriptional coactivator of the nuclear hormone  
5 receptors for the female sex steroids estrogen (ER $\alpha$ ) and progesterone. Furthermore, RIZ1 is required for normal growth and development of female target organs, such as the uterus, vagina, and mammary gland, in response to estrogen and progesterone. Therefore RIZ1, and  
10 particularly the PR domain of RIZ1, is a physiologically important regulator of progesterone and estrogen receptor activity. Compounds that modulate RIZ1 activity are thus likely to also be able to modulate progesterone receptor and/or ER $\alpha$  activity. Such compounds can be used as  
15 therapeutics to prevent, ameliorate or treat conditions that are benefitted by modulated female sex steroid receptor activity, including reproductive conditions and cancer.

In one embodiment, the invention provides a  
20 method of screening for a compound that modulates progesterone receptor activity. The method is practiced by providing a RIZ1 modulatory compound, and determining the ability of the compound to also modulate progesterone receptor activity.

25 As used herein, the term "RIZ1 modulatory compound" refers to a compound that affects a RIZ1 biological activity, such as a binding interaction with a cellular molecule or an enzymatic activity (e.g. histone methyltransferase activity). A RIZ1 modulatory compound  
30 can thus be, for example, a compound that selectively binds RIZ1, a compound that modulates (by either increasing or decreasing) an interaction between RIZ1 and a binding partner, a compound that modulates a functional

activity of RIZ1, or a compound with several of these effects.

Compounds that selectively bind RIZ1 are known in the art or can be identified by available screening  
5 methods. For example, compounds that selectively bind RIZ1 include RIZ1-specific antibodies (described, for example, in U.S. Patent No. 5,811,304) and RIZ1 binding partners. As used herein, the term "RIZ1 binding  
10 partner" is intended to refer to a cellular molecule that normally binds with high affinity to a RIZ1, such as cellular proteins, nucleic acid molecules, enzymatic substrates and cofactors, the RIZ1-binding domains from these molecules, and RIZ1-binding variants and analogs of these molecules.

15 A "variant" of a RIZ1-binding partner has substantially the same amino acid or nucleotide sequence as the native molecule (e.g. at least 70%, 75%, 80%, 90%, 95%, 98% or higher identity), and retains substantially the same binding activity as the native molecule. In the  
20 case of a polypeptide, the polypeptide can have one or more conservative or non-conservative amino acid substitutions, additions or deletions compared to the naturally occurring sequence. Methods of preparing and assaying the binding activity of variants of RIZ1-binding  
25 partners are well known in the art.

An "analog" of a RIZ1-binding partner has substantially the same core chemical structure as the  
native molecule, and retain substantially the same  
binding activity as the native molecule. Methods of  
30 preparing and assaying the binding activity of analogs of RIZ1-binding partners are well known in the art.



An exemplary cellular protein that binds RIZ1 is the retinoblastoma protein (Rb). U.S. Patent No. 5,811,304 describes the specific binding of RIZ1 to full-length Rb and to a C-terminal 56 kDa fragment of Rb that contains the Rb binding pocket (see also Buyse et al., Proc. Natl. Acad. Sci. USA 92:4467-4471 (1995)). Other cellular proteins that selectively bind RIZ1 include the estrogen receptor (ER $\alpha$ ) (see Abbondanza et al., Proc. Natl. Acad. Sci. USA 97:3130-3135 (2000) and Example I) and progesterone receptor (PR) (see Example I). Additionally, GATA-3 (Shapiro et al., Gene 163:329-330 (1995)), p53, SRC1 and p300 are exemplary cellular proteins that selectively bind RIZ1.

Nucleic acid molecules that selectively bind RIZ1 are described, for example, in U.S. Patent No. 5,811,304. Exemplary nucleic acid molecules to which RIZ1 selectively binds, through its zinc finger DNA binding motifs, contain GC-rich or Sp-1-binding elements.

Exemplary enzymatic substrates and cofactors that selectively bind RIZ1 include GTP (see U.S. Patent No. 5,811,304), zinc ions (which associate with the zinc finger motifs) and S-adenosyl-methionine (SAM).

SAM acts as a substrate and methyl group donor for RIZ1 methyltransferase activity. Analogs of SAM include S-adenosyl-L or D-homocysteine (SAH), and adenosyl-L-ethionine. Other binding compounds can include, for example, A-adenosyl-gamma-thio-alpha-ketobutyrate, S-adenosyl-L-homocysteine sulfoxide, methylthioadenosine (MTA), L-homocysteine, L-homoserine, Adenosine, Adenine, ATP, CAMP and methionine.

Therefore, any of the above compounds, or variants, analogs, agonists and antagonists of these

compounds, can be assayed for their ability to modulate progesterone receptor activity by the methods of the invention.

Compounds that modulate the interaction between  
5 RIZ1 and a binding partner are also known in the art or can be identified by available screening methods. For example, U.S. Patent No. 5,811,304 describes a 17 amino acid Rb binding peptide (101-118: T-pep) from the SV40 large T antigen as a compound that interferes with the  
10 interaction between RIZ1 and Rb. Other molecules that bind to a RIZ1 binding partner, or include a binding domain from a RIZ1 binding partner, are expected to be compounds that modulate the interaction between RIZ1 and a binding partner. Other compounds that can modulate the  
15 interaction between RIZ1 and a binding partner can contain a RIZ1 binding domain, and thus compete with full-length RIZ1 for the binding partner. RIZ binding domains include, for example, the LXXLL steroid receptor binding motif described herein, as well as the  
20 E1A-related region; the leucine-zipper; conserved regions 1 and 2; common epitope 1; zinc fingers; the GTPase domain; the SH3 domain; the SH3-binding domain; and the PMT (PR) domain, each of which is described in U.S. Patent No. 5,811,304.

25 Other compounds that selectively bind RIZ1, or that modulate the interaction between RIZ1 and a binding partner, can be identified by either manual or high-throughput screening assays, starting from a library of known or unknown compounds, as described above.

30 A binding assay can use a detectably labeled candidate compound and an unlabeled RIZ1 (and optionally an unlabeled binding partner). Alternatively, a binding assay can use an unlabeled candidate compound or binding

partner and a labeled RIZ1. Other appropriate combinations of labeled and unlabeled molecules can be determined by the skilled person depending on the assay format.

5           A variety of competitive and non-competitive binding assay formats for determining binding between molecules are known in the art. These assays include both solution-based methods and solid phase methods (e.g. molecules bound to plates, chips, affinity columns and  
10 the like). Binding assays are amenable to either manual or high-throughput automated screening of compounds. Two exemplary binding assays are set forth in Example IIA and IIB.

          Detectable labels can include, for example, a  
15 radioisotope, fluorochrome, ferromagnetic substance, or luminescent substance. Exemplary radiolabels useful for labeling compounds include  $^{125}\text{I}$ ,  $^{14}\text{C}$  and  $^3\text{H}$ . Methods of detectably labeling organic molecules, either by incorporating labeled amino acids into the compound  
20 during synthesis, or by derivatizing the compound after synthesis, are known in the art.

          RIZ1 functional activities are described herein or are known in the art. Exemplary activities include, for example, transcriptional activation (see, for  
25 example, U.S. Patent No. 5,811,304), transcriptional repression (see, for example, Xie et al., J. Biol. Chem. 272:26360-26366 (1997)), histone methyltransferase activity (see Example I) and hormone receptor coactivation (see Examples I and II).

30           Suitable assays for identifying compounds that modulate RIZ1 transcriptional activation, repression and coactivation function can be determined by the skilled

person. Such assays are generally based on co-expression of RIZ1 and an appropriate promoter-linked reporter gene in a cell, under conditions where a certain amount of transcription occurs, contacting the cell with the candidate compound, and determining whether there is a change (i.e. either an increase or decrease) in transcriptional activity. Transcription based assays are well known in the art, and readily amenable to high-throughput screening assays. Methyltransferase activity assays have been described above.

A compound that is identified as a RIZ1 modulatory compound by any of the above methods is then tested, in either an *in vitro* or *in vivo* assay, to determine whether it also modulates progesterone receptor activity. A compound that "modulates" progesterone receptor activity is intended to refer to a compound that either increases or decreases a PR biological activity.

The progesterone receptor is normally present in an inactive form in the cytoplasm; where it interacts with molecular chaperones, immunophilins, and heat shock proteins. The active PR binds progesterone and translocates to the nucleus where it binds as a transcription factor to canonical DNA transcriptional elements present in progesterone-regulated genes. As described herein, RIZ1 coactivates the transcriptional activity of PR. A compound that modulates progesterone receptor (PR) activity can thus be, for example, a compound that alters the interaction between PR and the molecules that normally hold it in an inactive form; alters the interaction between PR and progesterone; alters the interaction between RIZ1 and PR; or directly increases or decreases the transcriptional activity or specificity of PR toward PR-regulated genes.

Competitive and non-competitive binding assays have been described above with respect to RIZ1, and can be applied to identifying compounds that modulate PR interactions with progesterone, regulatory molecules or RIZ1. Assays that measure PR transcriptional activity can also be used to identify compounds that modulate PR activity. Conveniently, the progesterone response element (PRE) can be operatively linked to a reporter gene (e.g. luciferase, green fluorescent protein, beta-galactosidase and the like) and expressed in a cell, and an increase or decrease in expression of the reporter gene in response to a compound determined. Such an assay is described in Example I and IIC. Alternatively, a gene that is naturally induced by PR can be used in such assays, such as casein, and the mRNA or protein product detected.

Transcription-based assays, reporter genes, and methods for detecting gene expression, and methods of adapting these assays for high-throughput screening are well known in the art and described, for example, in Ausubel et al., Current Protocols in Molecular Biology, John Wiley and Sons, Baltimore, MD (2001).

Other assays for compounds that modulate progesterone receptor activity include *in vivo* assays that report PR function, such as the assays for uterine, vaginal and mammary development described in Example I and IID. A change in development of these organs in response to treatment of the animal with a particular compound is consistent with the compound having an effect on PR activity.

Once identified, compounds that modulate progesterone receptor activity can be formulated as pharmaceutical compositions and used to prevent or treat

reproductive and endocrinological disorders and cancers. Such disorders can include, for example: infertility, tubal disease, ovulatory defects, endometriosis, disruptions of the estrous cycle, disruptions of the menstrual cycle, polycystic ovary syndrome, ovarian hyperstimulation syndrome, endometrial and ovarian tumors, autoimmune disorders, ectopic pregnancy, teratogenesis, cancer of the breast, fibrocystic breast disease, galactorrhea, disruptions of spermatogenesis, abnormal sperm physiology, cancer of the testis, cancer of the prostate, benign prostatic hyperplasia, prostatitis, carcinoma of the male breast and gynecomastia. Other conditions in which increasing or decreasing progesterone receptor activity would be beneficial are known in the art or can be determined by the skilled person. The invention thus provides for the administration of a pharmaceutical composition containing a compound that modulates progesterone receptor activity, to prevent or ameliorate any of the disorders and conditions described above.

The pharmaceutical compositions of the invention, which include compounds that modulate RIZ1 HMT activity, and compounds that modulate progesterone receptor activity, may be administered alone or in combination with at least one other agent, such as stabilizing compound, which may be administered in any sterile, biocompatible pharmaceutical carrier, including, but not limited to, saline, buffered saline, dextrose, and water. The compositions may be administered to a patient alone, or in combination with other agents, drugs or hormones.

The pharmaceutical compositions utilized in this invention may be administered by any number of

routes including, but not limited to, oral, intravenous, intramuscular, intra-arterial, intramedullary, intrathecal, intraventricular, transdermal, subcutaneous, intraperitoneal, intranasal, enteral, topical, 5 sublingual, or rectal means.

In addition to the active ingredients, these pharmaceutical compositions may contain suitable pharmaceutically-acceptable carriers comprising excipients and auxiliaries which facilitate processing of 10 the active compounds into preparations which can be used pharmaceutically. Further details on techniques for formulation and administration may be found in the latest edition of Remington's Pharmaceutical Sciences (Maack Publishing Co., Easton, Pa.).

15           Pharmaceutical compositions for oral administration can be formulated using pharmaceutically acceptable carriers well known in the art in dosages suitable for oral administration. Such carriers enable the pharmaceutical compositions to be formulated as 20 tablets, pills, dragees, capsules, liquids, gels, syrups, slurries, suspensions, and the like, for ingestion by the patient.

Pharmaceutical preparations for oral use can be obtained through combination of active compounds with 25 solid excipient, optionally grinding a resulting mixture, and processing the mixture of granules, after adding suitable auxiliaries, if desired, to obtain tablets or dragee cores. Suitable excipients are carbohydrate or protein fillers, such as sugars, including lactose, 30 sucrose, mannitol, or sorbitol; starch from corn, wheat, rice, potato, or other plants; cellulose, such as methyl cellulose, hydroxypropylmethyl-cellulose, or sodium carboxymethylcellulose; gums including arabic and

tragacanth; and proteins such as gelatin and collagen.  
If desired, disintegrating or solubilizing agents may be  
added, such as the cross-linked polyvinyl pyrrolidone,  
agar, alginic acid, or a salt thereof, such as sodium  
5 alginate.

Pills, capsules, tablets and the like may  
further contain suitable coatings, such as concentrated  
sugar solutions, which may also contain gum arabic, talc,  
polyvinylpyrrolidone, carbopol gel, polyethylene glycol,  
10 and/or titanium dioxide, lacquer solutions, and suitable  
organic solvents or solvent mixtures. Dyestuffs or  
pigments may be added to the pills or coatings for  
product identification or to characterize the quantity of  
active compound, i.e., dosage.

15           Pharmaceutical preparations which can be used  
orally include push-fit capsules made of gelatin, as well  
as soft, sealed capsules made of gelatin and a coating,  
such as glycerol or sorbitol. Push-fit capsules can  
contain active ingredients mixed with a filler or  
20 binders, such as lactose or starches, lubricants, such as  
talc or magnesium stearate, and, optionally, stabilizers.  
In soft capsules, the active compounds may be dissolved  
or suspended in suitable liquids, such as fatty oils,  
liquid, or liquid polyethylene glycol with or without  
25 stabilizers.

Pharmaceutical formulations suitable for  
parenteral administration may be formulated in aqueous  
solutions, preferably in physiologically compatible  
buffers such as Hanks' solution, Ringer's solution, or  
30 physiologically buffered saline. Aqueous injection  
suspensions may contain substances which increase the  
viscosity of the suspension, such as sodium carboxymethyl  
cellulose, sorbitol, or dextran. Additionally,



suspensions of the active compounds may be prepared as appropriate oily injection suspensions. Suitable lipophilic solvents or vehicles include fatty oils such as sesame oil, or synthetic fatty acid esters, such as ethyl oleate or triglycerides, or liposomes. Non-lipid polycationic amino polymers may also be used for delivery. Optionally, the suspension may also contain suitable stabilizers or agents which increase the solubility of the compounds to allow for the preparation of highly concentrated solutions.

For topical or nasal administration, penetrants appropriate to the particular barrier to be permeated are used in the formulation. Such penetrants are generally known in the art.

The pharmaceutical compositions of the present invention may be manufactured in a manner that is known in the art, e.g., by means of conventional mixing, dissolving, granulating, dragee-making, levigating, emulsifying, encapsulating, entrapping, or lyophilizing processes.

The pharmaceutical composition may be provided as a salt and can be formed with many acids, including but not limited to, hydrochloric, sulfuric, acetic, lactic, tartaric, malic, succinic, etc. Salts tend to be more soluble in aqueous or other protonic solvents than are the corresponding free base forms. In other cases, the preferred preparation may be a lyophilized powder which may contain any or all of the following: 1-50 mM histidine, 0.1%-2% sucrose, and 2-7% mannitol, at a pH range of 4.5 to 5.5, that is combined with buffer prior to use.

After pharmaceutical compositions have been prepared, they can be placed in an appropriate container and labeled for treatment of an indicated condition. Such labeling can include amount, frequency, and method  
5 of administration.

Pharmaceutical compositions suitable for use in the invention include compositions wherein the active ingredients are contained in an effective amount to achieve the intended purpose. The determination of an  
10 effective dose is well within the capability of those skilled in the art.

For any compound, the therapeutically effective dose can be estimated initially either in cell culture assays, or in animal models, usually mice, rats, rabbits,  
15 dogs, or pigs. The animal model may also be used to determine the appropriate concentration range and route of administration. Such information can then be used to determine useful doses and routes for administration in humans.

20 A therapeutically effective dose refers to that amount of active ingredient which ameliorates the symptoms or condition. Therapeutic efficacy and toxicity may be determined by standard pharmaceutical procedures in cell cultures or experimental animals, e.g., ED50 (the  
25 dose therapeutically effective in 50% of the population) and LD50 (the dose lethal to 50% of the population). The dose ratio of toxic to therapeutic effects is the therapeutic index, and it can be expressed as the ratio, LD50/ED50. Pharmaceutical compositions which exhibit  
30 large therapeutic indices are preferred. The data obtained from cell culture assays and animal studies is used in formulating a range of dosage for human use. The dosage contained in such compositions is preferably

within a range of circulating concentrations that include the ED50 with little or no toxicity. The dosage varies within this range depending upon the dosage form employed, sensitivity of the patient, and the route of administration.

The exact dosage will be determined by the practitioner, in light of factors related to the subject that requires treatment. Dosage and administration are adjusted to provide sufficient levels of the active moiety or to maintain the desired effect. Factors which may be taken into account include the severity of the disease state, general health of the subject, age, weight, and gender of the subject, diet, time and frequency of administration, drug combination(s), reaction sensitivities, and tolerance/response to therapy. Long-acting pharmaceutical compositions may be administered every 3 to 4 days, every week, or once every two weeks depending on half-life and clearance rate of the particular formulation.

Normal dosage amounts may vary from 0.1  $\mu$ g to 100 mg, up to a total dose of about 1 g, depending upon the route of administration. Guidance as to particular dosages and methods of delivery is provided in the literature and generally available to practitioners in the art.

As disclosed herein, RIZ1 is an estrogen receptor coactivator. RIZ1 silencing is significantly more common in ER-negative breast cancers compared with ER-positive breast cancers, and appears to be implicated in the acquisition of estrogen resistance, since nearly half of ER+ cancer tissues examined were determined to be RIZ1 expression-negative.

The action of estrogen is mediated via interaction with a specific receptor (ER) that initiates a series of downstream events, leading to the modulation of hormone-responsive genes and cell proliferation.

- 5 Endocrine therapy, including both surgical and medical therapies to reduce estrogen action, is the preferred first-line therapy in patients with ER+ cancers.

However, a large percentage of patients with ER-positive tumors do not respond to endocrine therapy.

- 10 The determination that RIZ1 inactivation is highly associated with decreased estrogen responsiveness in breast cancer cells allows RIZ1 status to be used to predict whether an individual with an ER+ tumor is likely to respond to endocrine therapy.

- 15 Based on information regarding RIZ1 status, a clinician can determine a suitable treatment for the particular individual that is more likely to be effective in prolonging disease-free survival and/or reducing mortality, while avoiding exposing the individual to  
20 unnecessary treatments with potential side effects. For an individual determined by the invention method to have a normal likelihood of responding to endocrine therapy, endocrine therapy is appropriate. However, for an individual determined by the invention method to have a  
25 reduced likelihood of responding to endocrine therapy, an alternative treatment can be chosen from among the available treatment options. Alternatively, for such an individual, endocrine therapy can be combined with an additional treatment.

- 30 The nature of an alternative or additional treatment for such an individual can be chosen by the clinician depending on the type and stage of tumor, extent of metastasis, overall health of the individual

and other concurrent treatments. For example, in an individual determined to have a reduced likelihood of responding to endocrine therapy, chemotherapy, surgery or radiation can be combined with, or used as an alternative to, endocrine therapy.

Accordingly, the invention provides a method of identifying an individual with an estrogen receptor positive (ER+) tumor having a reduced likelihood of responding to endocrine therapy. The method is practiced by determining the RIZ1 status of the tumor, wherein an abnormal RIZ1 status identifies the individual as an individual with a reduced likelihood of responding to endocrine therapy. A normal RIZ1 status identifies the individual as an individual with a normal likelihood of responding to endocrine therapy.

As used herein, the term "endocrine therapy" refers to a therapy that reduces the proliferative potential of estrogen. Endocrine therapy can be either surgical or medical, and affect any aspect of estrogen action, including estrogen production and estrogen receptor signaling.

Methods of ablating estrogen production include surgical removal or irradiation of the ovaries, which are the primary source of estrogen in premenopausal women. Ablation of estrogen production can also be achieved pharmacologically by using luteinizing hormone-releasing hormone or gonadotropin-releasing hormone agonists.

Selective estrogen receptor modulators (SERMs) refer to compounds that blunt or block the effects of an estrogen agonist, such as  $17\beta$ -estradiol, when administered concomitantly in a test system. SERMs used in cancer therapy, although commonly known as "anti-

estrogens," generally exhibit a combination of anti-estrogenic and estrogenic effects. The antagonist effect of tamoxifen, the most widely used SERM, is a result of blocking the transmission of E2-ER-initiated signals for cell proliferation. Other SERMs include 4-hydroxytamoxifen and related triphenylene antiestrogens; clomiphene; and non-uterotrophic antiestrogens, such as raloxifene, droloxifene, idoxifene, nafoxidine, toremifene, TAT-59, levomeloxifene, LY-353381, CP-336156, MDL-103323, EM-800, ERA-923, ICI-182,780 and the like. Other SERMs are known in the art or can be determined.

Aromatase inhibitors are also used in endocrine therapy for ER+ cancers in postmenopausal women. Aromatase catalyzes the final step in the synthesis of the estrogens estrone and estradiol from androgens and, therefore, inhibitors of aromatase reduce estrogen production. Exemplary aromatase inhibitors include aminoglutethimide, megestrol acetate, anastrozole, letrozole and exemestane.

As used herein, the term "estrogen receptor positive (ER+) tumor" refers to a tumor that expresses estrogen receptors above an art-recognized threshold level. A number of assays are routinely used in the art to categorize a tumor as ER+ or ER-. Such assays include, for example, ligand binding assays (LBA) and immunohistochemistry (IHC). Conveniently, immunohistochemistry can allow for simultaneous determination of ER status and RIZ1 status of a tumor, as described herein. Generally, an ER level of greater than 3fmol/mg cytosolic protein by an LBA is considered ER+, whereas greater than 1% positive cells by an IHC assay is considered ER+ (Harvey et al., J. Clin. Oncol. 17:1474-1481 (1999); Elledge et al., Int. J. Cancer (Pred.

Oncol.) 89:111-117 (2000)). A cutoff of 10% positive cells was considered ER+ in the immunohistochemical assays described in Example I. However, the results would not have changed significantly using a 1% cutoff.

5           As used herein, the term "tumor" refers to a malignant neoplasm. A tumor can be either a primary lesion or a metastatic lesion. Tumors that have been shown to express estrogen receptors include breast carcinoma, endometrial carcinoma, prostate carcinoma,  
10 ovarian carcinoma, renal carcinoma, melanoma, colorectal tumors, desmoid tumors, pancreatic carcinoma, and pituitary tumors. The methods of the invention are applicable to ER+ tumors of any of these types, as well as other tumors determined to be ER+. The methods of the  
15 invention are applicable to ER+ cancers at any clinical stage, including localized cancers, cancers with regional spread and cancers with distant spread.

          Additionally, the method can be practiced with respect to individuals whose tissues upon biopsy are  
20 determined not to be malignant, including tissues that are determined to be normal or precancerous. An abnormal RIZ1 status in such ER+ tissues is predictive of an increased likelihood of the individual developing a tumor in that tissue. In particular, in women with low amounts  
25 of circulating estrogen, including post-menopausal women and women whose ovaries have been removed, an abnormal RIZ1 status in ER+ tissues is predictive of an increased likelihood of the individual developing a tumor in that tissue. For such individuals, prophylactic treatment,  
30 other than endocrine therapy, can be warranted. Suitable prophylactic treatments, including lifestyle changes (e.g. diet and exercise) and drug treatments, can be determined by those skilled in the art.

As used herein, the term "status" with respect to RIZ1 refers to a measurable property that is correlated with RIZ1 polypeptide function in the tumor. The measurable property that is determined can be a  
5 property of the RIZ1 polypeptide or of the encoding RIZ1 gene or mRNA.

An exemplary measurable property correlated with RIZ1 polypeptide function in the tumor in the tumor is the amount of the RIZ1 gene, mRNA or polypeptide in  
10 the tumor. An "abnormal RIZ1 status" in this case can thus be a decreased amount, in the tumor sample, of the RIZ1 gene, mRNA or polypeptide. Generally, a decreased amount of the RIZ1 gene, mRNA or polypeptide in the tumor refers to at least an 80% reduction, such as a 90%  
15 reduction, in the amount of the RIZ1 gene, mRNA or polypeptide in the tumor sample, relative to the amount in a control sample. The skilled person can determine an appropriate control for a particular assay, such as nearby normal tissue from the same individual, or a tumor  
20 sample previously identified as having a normal amount of the RIZ1 gene, mRNA or polypeptide.

A further exemplary measurable property correlated with RIZ1 polypeptide function in the tumor is the structural integrity of the RIZ1 gene. An "abnormal  
25 RIZ1 status" in this case can thus be a mutation in the normal nucleotide sequence of the RIZ1 gene in the tumor sample. Such a mutation can be determined directly by sequencing all or selected portions of the RIZ1 gene or mRNA. A mutation in the RIZ1 gene sequence can also be  
30 inferred indirectly, such as by detecting an alteration in the normal size or abundance of the RIZ1 mRNA, an alteration in the normal ratio of RIZ2/RIZ1, or by detecting an alteration in the normal size, stability or localization of the RIZ1 polypeptide product. Mutations



that can affect the ability of the RIZ1 polypeptide to be expressed or function normally include, for example, genomic deletions, frameshift mutations (Chadwick et al., Proc. Natl. Acad. Sci. USA 97:2662-2667 (2000); Piao et al., Cancer Res. 60:4701-4704 (2000); Sakurada et al., Genes, Chroms. Cancer 30:207-211 (2001)), missense mutations, nonsense mutations, and mutations in regulatory regions (U.S. Patent No. 5,811,304).

Methods for determining the amount or structural integrity of a particular gene, mRNA or polypeptide in a sample are well known in the art and described, for example, in Sambrook et al., Molecular Cloning: A Laboratory Manual, 3rd ed., Cold Spring Harbor Press, Plainview, New York (2001); Ausubel et al. (15) (Current Protocols in Molecular Biology (Supplement 47), John Wiley & Sons, New York (1999)). The particular method for a given application can be chosen by the skilled person. Exemplary methods include, for example, DNA sequencing, SSCP, Southern blotting, PCR, Northern blotting, RT-PCR, RNase protection, *in situ* hybridization, immunohistochemistry, immunoblotting and immunoprecipitation. Reagents suitable for detecting RIZ1 nucleic acid molecules in these methods, such as hybridization probes and PCR primers, are known in the art and described, for example, in He et al., Cancer Res. 4238-4244 (1998) and in the Example herein. Reagents suitable for detecting RIZ1 polypeptide in these methods, such as the KG7.1S and 2D7 antibodies, are also known in the art and described, for example, in Buyse et al. Proc. Natl. Acad. Sci. USA 92:4467-4471 (1995).

Another exemplary measurable property correlated with RIZ1 polypeptide function in the tumor is the extent of methylation of the RIZ1 gene promoter. As described in Du et al., Cancer Res. 61:8094-8099 (2001),

partial or complete methylation of the 35 CpGs in the RIZ1 promoter is strongly correlated with reduced RIZ1 mRNA expression, which in turn results in reduced RIZ1 polypeptide abundance. An "abnormal RIZ1 status" in this case can thus be partial or complete RIZ1 promoter CpG methylation.

RIZ1 promoter methylation can be determined, for example, by the methods described in Du et al., supra (2001). Briefly, one method involves treating genomic DNA from a sample with bisulfite, amplifying the RIZ1 promoter DNA using polymerase chain reaction (PCR), followed by cloning and sequencing the amplified product. Bisulfite treatment converts unmethylated cytosines to uridine, whereas methylated cytosines are resistant to conversion. An exemplary primer set suitable for RIZ1 promoter amplification by this method is 5'-GGTTGGGTGGTGGTTATTGGG-3' (SEQ ID NO:5) and 5'-CAAAACCGCCCTGCGCCACTCCTTACC-3' (SEQ ID NO:6). An alternative method involves treating genomic DNA from a sample with bisulfite and amplifying the DNA using methylation-specific polymerase chain reaction (MSP) with RIZ1 specific primers. An exemplary primer set for selectively amplifying methylated RIZ1 promoter DNA is 5'-GTGGTGGTTATTGGGCGACGGC-3' (SEQ ID NO:7) and 5'-GCTATTTGCGCCGACCCGACG-3' (SEQ ID NO:8), whereas an exemplary primer set for selectively amplifying unmethylated RIZ1 promoter DNA is 5'-TGGTGGTTATTGGGTGATGGT-3' (SEQ ID NO:9) and 5'-ACTATTTACCAACCCCAAGA-3' (SEQ ID NO:10).

Another exemplary measurable property correlated with RIZ1 polypeptide function in the tumor is histone methyltransferase activity of the RIZ1 polypeptide. An "abnormal RIZ1 status" in this case can thus be altered H1-K25 methylation activity, altered

H3-K9 methylation activity, or an alteration in the normal RIZ activity toward another identified histone methylation site. Such an alteration can be either increased or decreased HMT activity, relative to a normal control. Histone methyltransferase assays have been described above, and can be adapted by the skilled person for determining histone methyltransferase activity of a RIZ1 polypeptide in a tumor sample.

The skilled person can determine other measurable properties correlated with RIZ1 polypeptide function in the tumor, which can be used in the invention method to determine RIZ1 status. If by any of these criteria it is determined that the RIZ1 polypeptide would be unlikely to function normally in the tumor, whether because of altered amount, structure or activity, the tumor is designated as having an "abnormal RIZ1 status," and the individual identified as having a "reduced likelihood of responding to endocrine therapy." Accordingly, alternatives to endocrine therapy, or the use of endocrine therapy in combination with other therapies, are warranted in such an individual to improve the chance of disease-free and overall survival.

The following examples are intended to illustrate but not limit the present invention.

25

#### EXAMPLE I

This example shows that the tumor suppressor gene RIZ1(PRDM2) is a member of the histone methyltransferase superfamily and a coactivator of female sex hormone receptors. This example also shows that RIZ1 mediates female sex hormone action *in vivo*, and that loss of its function is involved in the transition to hormone

resistance and hormone independent growth of breast cancer.

### Materials and Methods

#### *Plasmids and transient transfections*

5           The human RIZ1 and RIZ2 expression vectors and RIZ1 mutant vectors used are described in Steele-Perkins et al., Genes Dev. 15:2250-2262 (2001). The mammalian RIZ1 expression vector p3RIZ1RH4.1 was constructed by cloning the full-length wild type human RIZ1 cDNA into  
10 the pcDNA3 vector (Invitrogen). The vector p3RIZ1RH4.1-L965A containing a missense mutation (leucine to alanine) was generated by the Quick-Change mutagenesis kit (Stratagene), and verified by DNA sequencing. Expression vectors of RIZ proteins and expression vectors  
15 containing full length mammalian ER $\alpha$ , PR, RAR $\alpha$ , RXR $\alpha$ , VDR, AR, TR and GR were cotransfected into CV-1 cells with an appropriate reporter construct containing a synthetic hormone response element linked to the tk-CAT reporter. The reporter TREpal-tk-CAT containing a  
20 synthetic response element for RAR, RXR and TR (Zhang et al., Nature 355:441-446 (1992)) was used to evaluate effects of RAR $\alpha$ , RXR $\alpha$ , and TR, the GRE-tk-CAT (Zhang et al., J. Biol. Chem. 266:8248-8254 (1991)) for GR and AR, the ERE-tk-CAT (Lee et al., Mol. Cell. Biol. 15:4194-4207  
25 (1995)) for ER $\alpha$ , the PRE-tk-cat for PR (McKay and Cidlowski, Mol. Endocrinol. 12:45-56 (1998)), and the VDRE-tk-CAT (Agadir et al., Carcinogenesis 20:577-582 (1999)) for VDR.

30           A calcium phosphate precipitation procedure was used for transient transfection as described previously (Lee et al., Mol. Cell. Biol. 15:4194-4207 (1995)). Briefly,  $0.5-1.0 \times 10^5$  cells/well were seeded in 24-well

plates, and 50-400 ng of RIZ plasmids, 100 ng of expression vectors for NHRs, 100 ng of reporter plasmid, and 100 ng of a  $\beta$ -gal expression vector were mixed with carrier DNA to 1  $\mu$ g of total DNA/well. Transfections of  
5 MCF-7 cells ( $2 \times 10^5$  cells/well in 6-well plates) used the Effectene Transfection Reagent (Qiagen), according to the manufacture's instructions. Cells were treated with or without the indicated hormone (50 nM for estradiol (E2), and 100 nM for R5020 (synthetic P agonist), all-trans  
10 retinoic acid (all-trans-RA), 9-cis retinoic acid (9-cis-RA), 1,25 dihydroxyvitamin D3 (VD3), dihydrotestosterone (DHT), triiodothyronine (T3), and dexamethasone (DEX); all from Sigma except R5020 which was from NEN) for 24 h. and CAT activity was measured as  
15 described (Lee et al., Mol. Cell. Biol. 15:4194-4207 (1995)). CAT values were normalized for transfection efficiency by the corresponding  $\beta$ -gal activity.

Plasmids used for GST protein production were constructed by PCR cloning as follows. To express human  
20 RIZ1 protein 1-200 residue region, full length human RIZ1 cDNA plasmid was used as template for PCR (Pfu polymerase, Stratagene) by primers hRP109.2 (AAA CCA TGG ATC AGA ACA CTA CTG AG (SEQ ID NO:11)) and RP274 (CCG TAA GCT TCA TGC AGA GGT GAA ATC TGG C (SEQ ID NO:12)). The  
25 PCR fragment was cloned into the NcoI and HindIII sites in the vector pKG-PBR (Huang et al., J. Biol. Chem. 273:15933-15940 (1998b)) to generate pKG-HN1. BL-21 cells were transformed by pKG-HN1 to produce the GST-RIZ200 protein. To express RIZ1 protein 1-161  
30 residue region, a stop codon was introduced at residue 162 in the plasmid pKG-HN1 by using the Quick-Change mutagenesis kit (Stratagene). The primers used were RP296P1 (CGA GCC AGC GCC CGG AGC TAA GCT TAA GCG GAG CTC CCC (SEQ ID NO:13)) and RP296P2 that has the complement  
35 sequence of RP296P1. To generate missense mutant

GST-RIZ200 proteins, mutagenesis by Quick-Change was performed on the pKG-HN1 plasmid template using primers as described previously (Steele-Perkins et al., Genes Dev. 15:2250-2262 (2001)). To generate longer peptide fragments of RIZ1 in bacteria, the N-terminal 520-residue fragment was first cloned into the NcoI and HindIII sites of pKG-PBR vector by PCR to generate the plasmid pKGH205. However, this plasmid did not give high yields of the expected protein product. Next, a stop codon was introduced into this plasmid by Quickchange mutagenesis to generate pKGRIZ332, which then produced high yields of the RIZ1 N-terminus 332-residue peptide. The PCR primers used for mutagenesis are: RP307: GAT TTA TTA GAG GAA tga AAA ACA ACT TCA GAA G (SEQ ID NO:14), and RP308: CTT CTG AAG TTG TTT TTc aTT CCT CTA ATA AAT C (SEQ ID NO:15).

#### *Immunoprecipitation and Immunoblot*

MCF-7 and T47-D breast cancer cells were grown in 15 cm dishes in DMEM with 5 % FCS, and 2 mM L-glutamine. Subconfluent cells ( $4 \times 10^6$ ) were then cultured in DMEM without phenol red, serum or hormones for an additional 3 days, during which media was changed twice daily. Cells were incubated for 24 h with or without hormones (50 nM for E2 and 100 nM for R5020) and infected with an adenovirus vector containing RIZ1 (AdRIZ1; at a concentration of  $1 \times 10^{10}$  viral particles/15 cm dish) or the empty vector (AdNull) (He et al., Cancer Res. 58:4238-4244 (1998)). Cells were then grown for an additional 48 h in DMEM without phenol red supplemented with 5 % charcoal-treated FCS (Omega Scientific). Cells were harvested and proceeded to immunoprecipitation and immunoblot essentially as described (Buyse et al., Proc. Natl. Acad. Sci. U.S.A. 92:4467-4471 (1995); Liu et al., J. Biol. Chem. 272:2984-2991 (1997)). Antibodies used included RIZ monoclonal antibody 2D7 (Buyse et al., Proc.

Natl. Acad. Sci. U.S.A. 92:4467-4471 (1995)), monoclonal antibodies versus ER $\alpha$  (sc-8005, Santa Cruz Biotechnology), progesterone receptor (1A6, DAKO). For histone methylation analysis, anti-dimethyl-H3-K9 and anti-dimethyl-H3-K4 antibodies (Upstate Biotech) were used for immunoblot analysis of acid extracted histones from cells infected with control and AdRIZ1 viruses for 48 hr. For H1 phosphorylation analysis, anti-phosph-H1 and anti-H1 antibody (Upstate Biotech) were used for immunoblot analysis of acid extracted histones.

#### *Mice*

RIZ1  $-/-$  mice were generated as described in Steele-Perkins et al., Genes Dev. 15:2250-2262 (2001). Briefly, a PR-domain-encoding cDNA fragment of rat RIZ1 cDNA was used to screen a mouse 129Sv genomic library (Stratagene) to obtain mouse genomic DNA. The targeting construct was designed to insert the PGK-neo<sup>r</sup>-BpA cassette (Soriano et al., Cell 64:693-702 (1991)) into exon 5 and to place the Herpes Simplex Virus TK cassette from PMC1-TK (Mansour et al., Nature 336:348-352 (1988)) external and adjacent to the region of homology. The targeting plasmid was linearized by XhoI digestion and electroporated into D3 ES cells (Gossler et al., Proc. Natl. Acad. Sci. USA 83:9065-9069 (1986)). The ES clones were selected in media containing G418 (150  $\mu$ g/ml) and ganciclovir (2.0  $\mu$ M). Homologous recombination events were screened by Southern blot analysis with XbaI digestion and hybridization to the 0.5-kb XbaI-BglIII 5' probe or with NotI plus EcoRI digestion and hybridization to the 0.5-kb SacII-NotI 3' probe. Five RIZ1 $+/-$  ES cell-lines were microinjected into C57BL/6 blastocysts that were implanted subsequently into the uteri of Balb/c foster mothers. Male chimeric mice generated from two RIZ1 $+/-$  ES clones transmitted the RIZ mutation into

germline. The presence of the RIZ1 mutant allele in the F1 animals was confirmed by Southern blotting and polymerase chain reaction (PCR) analysis of genomic DNA from the tail of animals. RIZ1+/- F1 animals were bred  
5 together to generate RIZ1-/- mice.

To examine reproductive functions, female RIZ1-/- mice were bred with male RIZ1+/- mice, female RIZ1+/- mice were bred with male RIZ1-/- mice, and breedings between heterozygous mutants were also  
10 performed. The number of litters was recorded, and all mice were routinely measured for body weight throughout the studies.

#### *Analysis of gene expression*

For RIZ staining, the RIZ rabbit antiserum  
15 KG7.1S (Buyse et al., Proc. Natl. Acad. Sci. U.S.A. 92:4467-4471 (1995)) was used at an optimal dilution of 1/200. For ER $\alpha$  staining the polyclonal rabbit anti-ER $\alpha$  antibody (sc-542 Santa Cruz) was used at a dilution of 1/250, and for PR staining the polyclonal rabbit anti-PR  
20 antibody (A0098 DAKO) was used at a dilution of 1/100, essentially as described (Tibbetts et al., Biol. Reprod. 59:1143-1152 (1998)). Tissues were fixed in Bouin's solution and processed by routine methods for embeddement in paraffin and sectioning (5  $\mu$ m). For RIZ and ER  
25 staining, sections were treated with trypsin (Zymed), whereas for PR staining, the sections were subjected to treatment using the Target Retrieval Solution (DAKO), all performed according to the manufacture's instructions. Sections were then incubated with the primary antibody  
30 followed with a biotinylated secondary antibody. The localization of the primary antibody was visualized with the imidazole-DAB reaction producing a brown colored stain, followed by hematoxyline counterstaining and



routine processing for bright-field microscopy analysis. Negative controls included omission of the primary antibodies and incubation with the preimmune KG7.1S antiserum, whereas colorectal xenograft tumors infected with AdRIZ1 were used as positive control for RIZ expression (Jiang and Huang, Cancer Res. 61:1796-1798 (2001)).

For RT-PCR analysis of RIZ gene expression, tissues from 7-week old RIZ1 +/- mice were pulverized in liquid nitrogen and total RNA was isolated using the TRIZOL reagent (Gibco BRL). cDNA was synthesized using the first strand cDNA synthesis kit (Gibco BRL). Oligos RP260 (5'-CTC ATT CAT CTA AGA AAG GTG G-3'; SEQ ID NO:16) + RP259 (5'-TGA TTC CAG GTC ACT TCA GG-3'; SEQ ID NO:17) and RP170 (5'-GAA GCC AAA GGC CTC TCA TC-3'; SEQ ID NO:18) + K05 (5'-AGA CTC TGG CTG AGG TAC C-3'; SEQ ID NO:19) were used in standard PCR conditions encompassing 30 cycles at an annealing temperature of 59°C to amplify the RIZ1+2 and RIZ1 specific fragments, respectively.

#### 20 *Analysis of target organs in response to hormone treatments*

To investigate E2-mediated increments in proliferation and hyperemia of uteri and changes in the cellular organization of the vaginal epithelium (Xu et al., Science 279:1922-1925 (1998)), 8-week old female RIZ -/- and RIZ +/- mice were ovariectomized (OVXed). At day 15-17 following OVX, mice were treated with S.C. injections of E2 (0.8 ng/g/day; Sigma) or with the vehicle (0.1 ml corn oil) alone for three days. The mice were then sacrificed at day 18 and the uterine wet weight was measured, and vaginal tissue was collected. The tissues were processed as above and vaginal specimens were stained with routine H & E and uterine specimens for

PR expression. The thickness of the vaginal epithelium and the cornified layer was photographed and measured as described in Figure 4 using the Spot 3.2.4. software (Diagnostic Instruments).

5 To evaluate the P- and E2-mediated decidual response a previously described protocol was used (Lydon et al., Genes Dev. 9:2266-2278 (1995)); Xu et al., Science 279:1922-1925 (1998)). Briefly, 8 week old female mice were OVXed and at day 10-12 following OVX,  
10 mice were treated with S.C. injections of E2 in corn oil (100 ng/day), then treated with P (1 mg/day; Sigma) + E2 (6.7 ng/day) from day 16-23. Six h after the third P + E2 injection on day 18, the left uterine horn (right horn served as a control) was traumatically stimulated by  
15 insertion of a burred needle proximal to the cervix and longitudinally scratching the entire length of the uterine horn antimesometrially. The mice were sacrificed on day 23, six hour after the last P + E2 injection, and the wet weights of the left and right uterine horns were  
20 measured.

To measure effects on mammapoiesis by normal development, pregnancy, and female sex hormone treatment the following protocols were used (Xu et al., Proc. Natl. Acad. Sci. USA 97:6379-6384 (2000); Xu et al., Science  
25 279:1922-1925 (1998)). To determine the effects of normal pubertal development on mammary gland growth, 7 week old virgin female mice were sacrificed. Additionally, pregnant female mice at day 19 of first pregnancy were sacrificed. To examine the effects of E2  
30 + P treatments on mammary gland development, 8 week old female mice were OVXed and at 14 days following OVX treated with S.C. 21-day releasing hormone pellets containing 0.1 mg of E2 and 10 mg P4 or S.C. placebo pellets (Innovative Research of America). On day 35, the

mice were sacrificed and the uterus was wet weighed and analyzed microscopically to ensure actual hormone release. In all cases, whole mounts were prepared from the fourth mammary gland and carmine-stained according to standard procedures (Evans et al., Oncogene 19:989-991 (2000)), and the relationship between mammary ducts and mammary fat pad, extent of branching as well as number of branches were investigated microscopically.

In order to evaluate the response to testosterone, twelve week old male mice were orchiectomized (ORCed) and testicles were wet weighed (Xu et al., Science 279:1922-1925 (1998)). Nine days following ORC, mice were treated during days 9-15 with S.C. injections of testosterone (3 mg/kg/day, Sigma) or vehicle alone (0.1 ml corn oil). On day 16, mice were sacrificed and the prostate and the pars prostate of the urethra (for technical reasons) were removed and wet weighed.

#### *Statistical analysis*

Student's unpaired t-test was used for statistical evaluation of means, with  $P < 0.05$  considered to be significant. All results are expressed as mean  $\pm$  SEM (standard error of the mean) or mean  $\pm$  SD (standard deviation, when stated). Fisher's exact test was used for evaluation of the difference between ER-negative and ER-positive breast cancers, with  $P < 0.05$  considered to be significant.

#### *Methylation assays*

Methylation reactions (30-40  $\mu$ l) contained 20 mM Tris-HCl (pH8.0), 0.2 M NaCl, 0.4 mM EDTA, affinity-purified GST-RIZ1 or immunoprecipitation

products, free histones (20-40 µg) purified from acid extraction of human cancer cells (A549 or U2OS), and 3 µl (1.65 µCi and 21 pmole) [methyl-<sup>3</sup>H]-adenosyl-methionine (Amersham-Pharmacia). Nuclear extracts were prepared according to standard procedures and used for immunoprecipitation. Antisera used for immunoprecipitation were anti-KG7.1S and preimmune serum as described previously (Buyse et al., Proc. Natl. Acad. Sci. U.S.A. 92:4467-4471 (1995)). Similar HMT activity has also been observed with immunoprecipitates using a different serum (data not shown), 1715, which was described previously (Steele-Perkins et al., Genes Dev. 15:2250-2262 (2001)). Various amount of SAH (Sigma) were added in some methylation reactions.

To determine the methylated residues on H3 and H1, H3 N terminus 1-20 residue peptides (Upstate Biotech), non-acetylated and acetylated on K9 and K14, and H1 N terminus 15-37 and 12-31 peptides were used as substrates for in vitro methylation reactions as described above. The reactions were resolved on Tris-Tricine 10-20% gel (Invitrogen) followed by fluorography. The <sup>3</sup>H-labelled H3 or H1 peptides were purified by HPLC and sequenced from the amino termini in an Applied Biosystems model 477A Protein Sequencer. After conversion, the samples were collected for determination of radioactivity by scintillation counting.

Histones from nocodazole treated cells were isolated by acid extraction of U2OS cells treated for 48 hour with 50 ng/ml nocodazole. Histones (20 mg) were treated with lambda protein phosphatase (400 U) (New England Biolabs) for 1 hr at 30 °C in buffer. Control histones were treated the same except that phosphatase was omitted from the incubation mixture.

## Results

*RIZ1 is a specific coactivator of ER and progesterone receptor*

RIZ1 has been shown to be a coactivator of  
5 estrogen receptor (ER) (Abbondanza et al., Proc. Natl. Acad. Sci. USA 97:3130-3135 (2000); Steele-Perkins et al., Genes Dev. 15:2250-2262 (2001)). To better understand the coactivator function of RIZ1, the effects of RIZ1 on ER were compared with its effects on several  
10 other NHRs. As shown in Fig. 1A, RIZ1 enhanced ER and progesterone receptor (PR) function in a ligand- and dose-dependent manner. In contrast, RIZ1 had no major effects on the activities of androgen receptor, retinoic acid receptor  $\alpha$  (RAR $\alpha$ ), retinoic X receptor  $\alpha$  (RXR $\alpha$ ),  
15 thyroid hormone receptor (not shown), glucocorticoid receptor (not shown), and vitamin D receptor (not shown). Similar to what has been found with ER (Steele-Perkins et al., Genes Dev. 15:2250-2262 (2001)), RIZ2 and RIZ1 missense mutants (C106Y and I188V) failed to coactivate  
20 progesterone receptor (Fig. 1A). The introduction of a point mutation in the LXXLL motif of RIZ also produced a protein unable to act as a coactivator for progesterone receptor (Fig. 1A). Consistent with RIZ1 effects on ER and progesterone receptor, protein complexes containing  
25 RIZ1 and these receptors were detected by a coimmunoprecipitation assay (Fig. 1B). Thus, RIZ1 is a specific coactivator of estrogen receptor and progesterone receptor but not for other nuclear hormone receptors, and the intact PR/SET-domain of RIZ1 is  
30 essential for its function.

To confirm the expected RIZ expression in the target organs of E2 and progesterone, RIZ expression was determined by immunohistochemistry. The gene products

displayed nuclear staining in the uterus, including both glandular and luminal epithelium as well as stromal and myometrial cells, similar to the expression of ER (Fig.1C). Additionally, RIZ is expressed in both mammary and vaginal epithelium. For these experiments, the serum anti-KG7.1S, which recognizes both RIZ1 and RIZ2, was used (Buyse et al., Proc. Natl. Acad. Sci. USA 92:4467-4471 (1995); Liu et al., J. Biol. Chem. 272:2984-2991 (1997)). Positive results from this antibody likely reflect expression of both proteins because they are coexpressed in most tissues (Buyse et al., Proc. Natl. Acad. Sci. USA 92:4467-4471 (1995); Liu et al., J. Biol. Chem. 272:2984-2991 (1997)). Nonetheless, by RT-PCR analysis using primer sets that can differentiate RIZ1 from RIZ2, it was confirmed that RIZ1 was indeed expressed in various target tissues of female sex steroid hormones (Fig. 1D).

*Partial hormone resistance in target organs of RIZ1 deficient female mice*

The RIZ1 mutant mice are deficient in RIZ1, but not RIZ2, and therefore lack specifically any PR-domain related functions of the gene (Steele-Perkins et al., Genes Dev. 15:2250-2262 (2001)). These animals are viable and fertile and show no gross developmental defects (Steele-Perkins et al., supra (2001)). To determine the physiological role of RIZ1 in steroid hormone action, uterine growth in response to E2 was first examined in ovariectomized (OVXed) mice (Lubahn et al., Proc. Natl. Acad. Sci. USA 90:11162-11166 (1993)). Wild-type or heterozygous RIZ1 mutant mice responded to E2 treatment with a  $5.3 \pm 0.5$  and  $5.4 \pm 0.4$  fold increase in uterine wet weight. Uteri of homozygous RIZ1 mutants showed a smaller increase of about  $3.7 \pm 0.4$  fold ( $P < 0.001$  for +/+ vs. -/-; Fig. 2A and B). Uterine

response to mechanical traumatization (decidual stimulation) is mainly a progesterone receptor-dependent process (Lydon et al., Genes Dev. 9:2266-2278 (1995)). OVXed mice were treated with a high dose of progesterone and a low dose of E2, followed by mechanical stimulation of the left uterine horn of each animal. The unstimulated left uterine horn served as a control. The decidual response resulted in an increase in the uterine horn size and was consistently observed in the stimulated left uterine horn in wild-type mice. Only a partial response was observed in the uterine horn of the RIZ1 null mutant (Fig. 2C). These results suggest that RIZ1 is required for maximal uterine response to E2 and progesterone in vivo.

Vaginal cornification in response to a three-day treatment of E2 was examined in OVXed mice. The hormone produces an increased vaginal thickening and cornification of the epithelium (Lubahn et al., supra (1993)), which was consistently seen in the wild-type mice (Fig. 2D,E). RIZ1 null female mice, however, failed to show a maximal such response, thus suggesting that RIZ1 is required for vaginal response to E2 in vivo.

The epithelial and stromal compartments of the uterus were then analyzed for E2-induced changes in progesterone receptor expression. Such a regulation via ER is highly compartment-specific and mimics the changes in the uterus during the estrous cycle (Tibbetts et al., Biol. Reprod. 59:1143-1152 (1998)). Progesterone receptor staining in OVXed RIZ1 +/+ and -/- mice treated with vehicle was apparently similar and showed strong immunoreactivity in almost all cells of both the luminal (LE) and glandular epithelium (GE), whereas only a fraction of stromal and myometrial cells were positive (Fig. 2G, data not shown). As expected, E2 treatment of

wild type animals had dual effect on progesterone receptor expression, reducing the levels in the LE and increasing the levels in the stromal and myometrial compartments. Immunoreactivity in the GE was unchanged, consistent with previous findings showing that E2 alone does not regulate progesterone receptor expression in this compartment (Fig. 2F,G) (Tibbetts et al., supra (1998)). Upon E2 treatment of RIZ1 deficient animals, the decrease of progesterone receptor levels in the LE was nearly 8-fold less than in wild type tissues, and the increase of the receptor level in the stroma and myometrium was nearly 2-fold less than normal. These findings suggest that RIZ1 plays a role in the E2 regulation of progesterone receptor expression in the uterus.

Steroid hormones play an important role in mammapoiesis (Hennighausen and Robinson, Genes Dev. 12:449-455 (1998)). Both E2 and progesterone are essential for alveolar development during pregnancy. In 7-week-old wild-type females, mammary ducts had started to grow into the mammary fat pad but with no apparent differences in the age-matched RIZ1 null mutants (Fig. 3A and B). However, when stimulated by pregnancy, alveolar structures in wild-type mammary glands were highly developed and appeared on all ductal sections, filling the interductal spaces. In the RIZ1 mutant mammary glands, alveoli were much less developed in terms of number and size of alveoli, and fewer alveoli were observed at the ends of ducts at the same stage of pregnancy (Fig. 3C through F). Although mammary glands of RIZ1 null mutants can still produce milk, these results suggest that RIZ1 is required for normal mammary ductal elongation and alveolar development in vivo during pregnancy. Mammary gland development in response to E2 and progesterone treatment in OVXed adult mice was also



examined. E2 and progesterone stimulated a complex ductal arborization and extensive alveolar formation in mammary glands of wild-type mice, which mimics a stage of mammapoiesis in early pregnancy. In the mammary glands  
5 of RIZ1 mutant mice, only partial ductal growth was observed after hormone treatment (Fig. 3G through J). Thus, RIZ1 seems to be required for efficient proliferation and differentiation of the mammary gland in response to E2 and progesterone.

10 *Normal steroid hormone response in RIZ1 deficient male mice*

Although RIZ1 is expressed in target tissues of male steroid hormones such as testis (Buyse et al., Proc. Natl. Acad. Sci. USA 92:4467-4471 (1995)), it does not  
15 appear to serve as coactivator for androgen receptor (Fig. 1A). To study this further, prostate growth was measured in orchiectomized male mice after they were treated with androgen. Similar to female mice, no difference in total body weight was noted in RIZ1 null  
20 mutant male animals (Table 1).

Table 1

		Mean $\pm$ S.D. (number of mice weighed)		
		5 weeks	8 weeks female	12 weeks female
RIZ1-/-	Female -			
	16.83 $\pm$ 1.64 (11)	83	21.28 $\pm$ 2.70 (32)	24.27 $\pm$ 2.21 (13)
	Male -			
	19.24 $\pm$ 2.25 (7)			
RIZ1+/-	Female -			
	16.83 $\pm$ 2.01 (15)		20.49 $\pm$ 2.70 (12)	24.51 $\pm$ 3.22 (23)
	Male -			
	20.00 $\pm$ 2.56 (15)			
5 RIZ1+/+	Female -			
	18.52 $\pm$ 1.55 (5)		20.37 $\pm$ 2.13 (31)	21.71 $\pm$ 3.49 (13)
	Male -			
	20.61 $\pm$ 2.72 (7)			

Eight days after orchiectomization, prostates in both wild-type and mutant animals regressed. Injection of testosterone for 7 days stimulated prostate growth in wild-type animals as well as in RIZ1 null animals. Wild-type and RIZ1 null mutant animals had similar ratios of the weight of prostate and urethra to body weight ( $1.19 \pm 0.03$  vs.  $1.14 \pm 0.03$ ;  $n=10$  and  $9$  mice for  $+/+$  and  $-/-$ , respectively;  $p=0.23$ ). They also had similar ratios of testis weight to body weight ( $7.7 \pm 0.4$  vs.  $7.4 \pm 0.4$ ;  $n=14$  for both genotypes;  $p=0.58$ ). Thus, tissue responses to testosterone are not significantly affected in mice lacking RIZ1, consistent with the lack of effect of the gene on androgen receptor transactivation functions (Fig. 1A).

#### 20 *Reduced litter size by RIZ1 deficient female mice*

The reproductive abilities of RIZ1 mutant mice were assessed. Female RIZ1-/- mice were bred with male RIZ1+/- mice and as controls, female RIZ1+/- mice were bred with male RIZ1-/- mice. Also, breedings between

heterozygous mutants were conducted. The average litter size from breeding RIZ<sup>-/-</sup> female x RIZ1<sup>+/-</sup> male was 6.6 ± 3.2 (S.D.; n = 35 litters) but the average litter size was 8.4 ± 2.0 (n = 20 litters) for litters of breeding RIZ1<sup>+/-</sup> female x RIZ1<sup>-/-</sup> male (P < 0.01), and 8.0 ± 2.2 (n = 50 litters) for litters of breeding between heterozygous mice (P < 0.02). The results indicated a slightly compromised reproductive ability associated with the female, but not male, mice deficient in RIZ1, consistent with the above results showing defective tissue responses to steroids in female but not male animals.

*Regulation of ER-dependent transcription in breast cancer by RIZ1 and RIZ2*

The above results suggest a physiological role of RIZ1 in E2 response of normal tissues. In view of RIZ1's tumor suppressor role, the question was posed whether RIZ also plays a role in the E2-response of breast cancer cells. RIZ1 is often lost but RIZ2 is uniformly present in breast cancer (He et al., Cancer Res. 58:4238-4244 (1998); Jiang and Huang, Histol. Histopathol. 15:109-117 (2000)). RIZ2 does not serve as a coactivator of ER and can actually neutralize the coactivator function of RIZ1 (Steele-Perkins et al., Genes Dev. 15:2250-2262 (2001)). Therefore, the effects of overexpressing RIZ1 and RIZ2 in MCF-7 were examined in breast cancer cells that express ER and RIZ1 (He et al., supra (1998)). When cotransfected with the ERE-tk-CAT reporter into MCF-7 cells, RIZ1 enhanced estrogen-induced reporter activity (Fig. 4A). In contrast, E2 induced reporter gene transcription was suppressed by RIZ2 expression. In CV-1 cells that lack ER, reporter gene expression was not affected by RIZ1, RIZ2, and E2 (Fig. 4B). The data suggest that RIZ1 can sensitize breast

cancer to E2 and that decreasing RIZ1 activity, as achieved by RIZ2 expression, can inhibit E2 sensitivity of breast cancer.

*RIZ1 gene silencing in hormone resistant breast cancer*

5                   If RIZ1 appears to facilitate E2-mediated normal and breast cancer growth as indicated by the above studies, why is it also a tumor suppressor and often silenced in breast cancer (He et al., supra (1998))? Upon re-analysis of the published data, it was found that  
10 RIZ1 loss is related to hormone resistance (as represented by ER status) among those breast cancer cell lines examined (Table 2).

                  A methylation-specific PCR assay was developed to examine RIZ1 promoter methylation, which showed that  
15 DNA methylation is a good indicator of lost or decreased RIZ1 mRNA expression in tumor cell lines and tissues (Du et al., Cancer Res. 22:8094-8099 (2001)). Methylation of RIZ1 promoter was found to be common in breast cancer tissues, occurring in 11 of 25 breast cancer tissues  
20 examined. Except for BT20 and ZR75-1 cell lines, the mRNA expression data in Table 2 are from (He et al., supra (1998)). Expression levels below 10% of normal were scored negative (-).

Table 2

	RIZ1		ER
	Methylation	mRNA	
5	MB468	+	-
	MCF-7	-	+
	MB-435	+	-
	T47-D	-	+
	MB231	+	-
	BT474	-	+
10	SKBR3	+	-
	BT549	-	+
	BT20	-	+

To address the relationship between RIZ1 and hormone responsiveness of breast cancer tissues, the ER expression status was examined for 18 of the 25 samples for which tissue sections were available. ER expression was determined by immunohistochemistry (below 10% positivity was considered negative) and was scored in a double-blind fashion without knowledge of the methylation status of these samples. The results showed the same trend as previously found in cell lines that ER negative tumors tend to be RIZ1 negative (Table 3).

Table 3

Expression	ER (-)		ER (+)	
	RIZ1 (-)	RIZ1 (+)	RIZ1 (-)	RIZ1 (+)
# tumor cases	83% (5/6)	17% (1/6)	42% (5/12)	58% (7/12)
# cell lines	67% (4/6)	20% (2/6)	0%	100% (4/4)
# Total	75% (9/12)	25% (3/12)	31% (5/16)	69% (11/16)

Thus, 5 of 6 ER-negative cases were RIZ1 negative compared to 5 of 12 ER-positive cases. Together with data from cell lines, these results shows that there is a significant association between RIZ1 silencing and ER-negative breast cancers (RIZ1 silencing found in 9 of 12 ER-negative samples versus 5 of 16 ER-positive samples,  $P < 0.05$ ). The results also reveal that nearly half of ER-positive cancer tissues were RIZ1 negative (5 of 12), indicating a potential role of RIZ1 silencing in the development of hormone resistance of ER-positive breast cancers. Together, these studies indicate a dual status of RIZ1 in breast cancer that is linked with the presence or absence of ER, and in turn, the early or late stage of the disease. The dual status of the gene appears consistent with its dual role as a tumor suppressor and an ER/E2-dependent tumor promoter (see discussion below).

*Histone methyltransferase activity of RIZ1 and regulation by estrogen*

The results of animal knock out studies presented here and previously (Steele-Perkins et al.,  
5 supra (2001)), as well as the dual role of RIZ1 in breast cancer discussed above, suggest a physiological role of RIZ1 as an ER-coactivator/growth promoter and a tumor suppressor. Because it is only the PR domain of RIZ1 that is missing in these animals or in tumors, owing to  
10 normal expression of RIZ2, it was asked whether RIZ1 has an activity that may explain its dual function as an ER-coactivator/growth promoter and tumor suppressor.

Recombinant GST fusion proteins of RIZ1's N-terminus PR domain region were prepared in bacteria.  
15 The recombinant proteins were purified and assayed for histone methyltransferase activity using histones as substrates and S-adenosyl-[methyl-<sup>3</sup>H]-L-methionine as methyl group donor. Reaction products were separated by SDS-PAGE and visualized by flurography. Specific  
20 labeling of histone H4 was observed in the presence of purified GST product consisting of residues 1-200 of RIZ1 (Fig. 5A). GST alone or a protein consisting of residues 1-161 lacked HMT activity. Furthermore, missense mutations found in tumors, C106Y, I188V, and A159V,  
25 abolished HMT activity. The results suggest that RIZ1 has HMT activity, which requires the conserved PR-domain (residues 30-160) as well as the non-conserved residues C-terminal to the PR-domain (residues 162-200).

It was next asked whether the HMT activity of  
30 the PR domain may be modulated by additional sequences of RIZ1 by testing longer RIZ1 N-terminal fragments made in bacteria. A GST fusion protein expressing the N-terminal 332-residue peptide showed similar activities to the

N-terminal 200-residue peptide in methylating H4 (Fig. 5B). An attempt to test an even longer peptide (520 residue) in bacteria was not successful because of low yield of protein. The result suggests that residues  
5 200-332 may not intrinsically contribute to HMT activity but could not exclude other regions of RIZ1 (333-1719) from playing role in modulating the HMT activity of the N-terminal 200-residue region.

A domain located at the C-terminus of RIZ1  
10 protein (amino acids 1514-1680) was previously defined that can bind to the PR domain of RIZ1 (Huang et al., J. Biol. Chem. 273:15933-15940 (1998)). It was next asked whether this domain, termed PBD or PR-binding domain, can regulate the PR HMT activity. The previously described  
15 GST fusion protein, GST-hRIZ1(1514-1680) or now termed GSTRIZPBD, was examined for its effects on the HMT activity of GSTRIZ332. Unexpectedly, GSTRIZPBD protein alone showed HMT activity, specifically on H4, suggesting that the RIZ PBD domain represents a novel catalytic  
20 motif of methyltransferase. A synergistic enhancement (more than additive effects) of HMT activity was observed when GSTRIZ322 and GSTRIZPBD were both present in HMT reactions, as indicated by the increased methylation of H4 and the methylation of a new substrate with a  
25 molecular weight similar to that of H1 (Fig. 5C). This result suggests that the PR domain HMT activity can be modulated by other regions of RIZ1, such as the PBD domain.

Enzyme activity of full-length RIZ1 protein  
30 from mammalian cells was next assayed. Human osteosarcoma U2OS cells, which express little RIZ1 (He et al., Cancer Res. 58:4238-4244 (1998)), were infected with AdRIZ1 virus or control AdNull virus and nuclear extracts from virus infected cells were immunoprecipitated with



RIZ serum anti-KG7.1S or preimmune serum.

Immunoprecipitated products by RIZ1 serum from RIZ1-expressing extracts caused methylation of H1, H3, and H4 (Fig. 6A). A similar result was also obtained using a different RIZ1 antiserum, 1715 (data not shown). As controls, HMT activity was not detected for immunoprecipitates of preimmune serum or when RIZ1 negative nuclear extracts were used for immunoprecipitation.

10               The data indicate that RIZ1 protein of mammalian cells has HMT activity, which is consistent with the activity of truncated proteins obtained from bacteria (Fig. 5). However, full length protein appears to target more histone substrates than truncated proteins from bacteria, probably because it has sequences or  
15               modifications or both that are missing in bacteria expressed proteins. The results are consistent with other regions of RIZ protein regulating PR domain HMT activity.

20               In view of RIZ1's role in tumor suppression, it was determined whether S-adenosylhomocysteine (SAH), an analog inhibitor of SAM-dependent methyltransferases whose abnormal accumulation is linked with  
                  carcinogenesis, can inhibit RIZ1 enzyme activities. The  
25               effect of SAH on the in vitro methylation of histones by immunoprecipitated RIZ1 was tested. A methylation assay was performed in the presence of 0, 0.5 and 5  $\mu$ M of SAH and 0.42  $\mu$ M of 3H-SAM. Histone methylation by RIZ1 was partially inhibited by 0.5  $\mu$ M of SAH and completely  
30               inhibited by 5  $\mu$ M of SAH (Fig. 6B). The results show that SAH is an inhibitor of RIZ1 methyltransferase.

                  The methylated lysine residues at position 4 and 9 of histone H3 are the best-characterized histone

lysine methylations with regard to their biological function. Methylation of H3-K9 is associated with gene repression and heterochromatin formation, whereas methylation of H3-K4 is often linked to gene activation  
5 (Noma et al., Science 293:1150-1155 (2001); Rea et al., Nature 406:593-599 (2000); Strahl et al., Curr. Biol. 11:1-5 (2001)). Each of the two lysine residues can be targeted by several different SET domain HMTs, which are  
10 non-H3 substrates such as H1 (Nishioka et al., Genes & Dev. 16:479-489 (2002); Rea et al., Nature 406:593-599 (2000); Tachibana et al., J. Biol. Chem. 276:25309-25317 (2001); Wang et al., Mol. Cell. 8:1207-1217 (2001a)). To determine whether RIZ1 may belong to such a group of  
15 H3-K4 or H3-K9 specific HMTs may immediately explain how RIZ1 functions in transcription. Therefore, it was determined whether RIZ1 may have any activity toward H3-K4 or H3-K9. First, it was determined whether RIZ1 can alter the methylation of these residues in vivo in  
20 mammalian cells. Histones were extracted from U2OS cells infected with AdRIZ1 or control virus and analyzed by western blot analysis using antibodies that recognize specifically H3-K9 methylation or H3-K4 methylation. The result shows that RIZ1 increased H3-K9 methylation but  
25 did not affect H3-K4 methylation (Fig. 6C), consistent with H3-K9 but not H3-K4 as a potential target of RIZ1 methyltransferase.

To show that RIZ1 can directly methylate H3-K9, peptide methylation and sequencing experiments were  
30 performed. A peptide consisting the N-terminal 1-20 residues of H3 was indeed methylated in vitro by immunoprecipitated RIZ1 protein using S-adenosyl-[methyl-3H]-L-methionine as methyl donor (Fig. 6D). In contrast, a peptide containing acetylated K9 and  
35 K14 was not methylated, consistent with K9 but not K4 as

a target of RIZ1. The migration of the peptide as a doublet has also been observed by others, and may be due to the poor resolution of SDS-PAGE. The methylated peptide was next purified by HPLC and subjected to peptide sequencing analysis. Purified peptides were sequenced from the amino termini and the radioactivity of each of the sequenced residues was determined by scintillation counting. A single radioactive peak at lysine 9 was observed (Fig. 6E). The results suggest that H3-K9, but not H3-K4, is a target of RIZ1 methyltransferase.

Next, the possible methylation site on H1 was determined. A H1 peptide (aa 15-37) was methylated by RIZ1 in vitro, which was then subjected to HPLC purification and sequencing analysis. Repeated methylation and sequencing analysis showed that lysine 25 was methylated by RIZ1 (Fig. 6F).

The capacity to methylate H3 in a K9 specific manner suggests a role for RIZ1 in transcriptional repression, which appears to be in conflict with RIZ1's role as an ER coactivator. To help understand this apparent paradox, the estrogen effects on RIZ1 HMT activity were examined. Nuclear extracts from control and E2 treated MCF-7 cells were immunoprecipitated by RIZ1 serum anti-KG7.1S or preimmune serum. As expected, HMT activity (labeling of H3 and H1) was detected in the immunoprecipitates of RIZ1 serum but not preimmune serum (Fig. 7A, lanes 1 and 3). Interestingly, estrogen treatment decreased H3 methylation but maintained or slightly enhanced H1 methylation by RIZ1 (Fig. 7A, lane 4). RNase protection analysis did not reveal altered RIZ1 or RIZ2 gene expression by E2 treatment of MCF-7 cells (not shown). Furthermore, E2 also similarly regulated the HMT activity of overexpressed RIZ1 protein

in AdRIZ1 virus infected MCF-7 cells (Fig. 7A, lanes 5-6); cells infected with control virus showed similar results as non-infected cells (not shown). Thus, E2 appears to regulate RIZ1 HMT activity in a selective way, 5 inhibiting specifically methylation of H3 but not H1. The data suggest that E2 may turn RIZ1, possibly with a dual role of transcriptional repressor and activator, into a transcriptional activator by selectively turn off its repressor activity as represented by H3 methylation 10 while enhancing its putative activator activity, presumably represented by H1 methylation.

Although the function of H1 methylation remains to be characterized, the results here suggest, at least, that RIZ1 methylation of H1 is unlikely to be similar to 15 H3-K9 methylation, in view of the contrasting behavior of these two methylation activities of RIZ1. Because H1 phosphorylation has been linked with transcriptional activation (Bhattacharjee et al., Mol. Cell. Biol. 21:5417-5425 (2001); Dou and Gorovsky, Mol. Cell. 6:225- 20 231 (2000)), it was next asked whether E2 or RIZ1 can enhance H1 phosphorylation. Histones were extracted from MCF7 cells infected with AdRIZ1 or control virus and analyzed by western blot analysis using antibodies that recognize specifically phosphorylated H1. The result 25 shows that both E2 and RIZ1 increased H1 phosphorylation (Fig. 7B), suggesting that E2 or RIZ1 may function to stimulate transcription in part by mediating enhanced H1 phosphorylation. That RIZ1 methylation of H1 was positively correlated with H1-phosphorylation suggests 30 that such methylation is correlated with transcriptional activation.

Next, the methyl acceptor potential of phosphorylated H1 with non- or hypophosphorylated H1 was compared. Histones were isolated from cells arrested at

G2/M by nocodazole, which were enriched for phosphorylated H1, and used as substrates for in vitro methylation by RIZ1. H1 from nocodazole treated cells appeared to serve as a poor substrates compared to H1  
5 from control cells (not shown). This result indicates either that H1 isolated from nocodazole treated cells was both phosphorylated and methylated or that phosphorylation of H1 may prevent its methylation. To resolve this, histones were treated with phosphatase  
10 prior to use as methylation substrates. Phosphatase treatment removed a significant amount of phosphate on H1 but did not affect its ability to serve as methylation substrates (not shown). Together, these results suggest that phosphorylated H1 is associated with a higher extent  
15 of methylation than non- or hypophosphorylated H1 and that methylated H1 is associated with a higher degree of phosphorylation than non- or hypomethylated H1.

The time course response of RIZ1 methylation activity to E2 treatment was then studied. A decrease of  
20 RIZ1 H3 methylation activity was found at 4 hr post E2 treatment, which stayed at low levels thereafter (not shown). Because E2-induced cell proliferation is not known to occur prior to at least 8 hr of treatment, the data suggest that inhibition of RIZ1 H3-methylation  
25 activity by E2 is unlikely a result of cell proliferation.

The observation that E2, a growth factor, has a sustained inhibitory effect on RIZ1 methylation of H3 but not H1 suggests that H3 and H1 methylation activity of  
30 RIZ1 may be oppositely linked with cell growth. To further study the contrasting behavior of H1 and H3 methylation, regulation of RIZ1 HMT activity by cell growth was examined, using 12-O-tetradecanoyl-phorbol-13-acetate (TPA) treatment of

U937 myeloid leukemic cells as a model system. U937 cells undergo growth arrest and terminal differentiation into monocytes when exposed to TPA. This cell model was selected for study because it has been shown in these 5 cells that RIZ gene expression is only marginally regulated by TPA. A slight increase (less than 2 fold) in RIZ1 mRNA, but no change in RIZ2 levels, was not observed until after 3 or more days of TPA treatment (Gazzerro et al., Mol. Med. 7:552-560 (2001)). Thus, any 10 significant changes in HMT activity, especially a decrease, may not be attributed to changes in RIZ1 protein amounts, at least within the first three days of treatment. The TPA effects on U937 cell growth and differentiation were confirmed by counting cell numbers 15 and examining cell morphology (not shown). It was also confirmed that RIZ gene expression was not significantly altered within the first 3 days of TPA treatment. By assaying the HMT activity of RIZ1 immunoprecipitates from nuclear extracts, it was found that H3 methylation was 20 increased, whereas H1 methylation was slightly decreased at 24 hr following TPA treatment (not shown). Similar results were observed at 3 days or 7 day post TPA treatment, although H3 methylation activity was noted to be lower than that at 1 day post treatment. No 25 significant change in HMT activity was noted for any treatment periods of less than 24 hr. The change at 24 hr post TPA treatment was well before any significant growth arrest had taken place, suggesting that such a change is unlikely a result of growth arrest. The 30 results suggest that the H3 methylation activity of RIZ1 is linked with growth arrest, whereas its H1 methylation activity is associated with cell proliferation, confirming the above described contrasting behavior of these activities in response to E2.

Discussion

The above results show that female mice deficient in RIZ1 display an impaired response to E2 and progesterone, and reduced reproductive abilities. RIZ1 shows coactivator activity for ER and progesterone receptor but no or weak activity for other NHRs examined. Furthermore, the results suggest that a coactivator (RIZ1) may serve as both an E2-dependent growth promoter in early stage breast cancer and a tumor suppressor in preventing breast cancer progression to an estrogen independent state. Estrogen mediated transactivation in breast cancer cells is suppressed by a RIZ1 antagonist (RIZ2), and hormone resistant breast cancers more commonly carry a silenced RIZ1 gene than hormone sensitive breast cancers. These data suggest that RIZ1 has HMT activity, which is mediated by two independent but interacting catalytic motifs. The HMT activity of the PR domain is linked to both its ER-coactivator function and tumor suppressor function. Cancer-associated mutations in the PR-domain, which inactivate its coactivator function, impair the HMT activity. Also, the HMT activity of RIZ1 can be regulated by E2, in a fashion consistent with RIZ1's role as an ER-coactivator.

The specificity of RIZ1 in its interaction with female sex NHRs contrasts to members of the SRC-1/p160 family members, which seem more promiscuous in this respect. Thus, one may expect more subtle phenotypes for RIZ1 deficient mice versus SRC-deficient animals. Indeed, this is the case even when comparing to mice deficient in SRC-1, which shows the mildest phenotypes among animals deficient in a SRC member (Wang et al., Proc. Natl. Acad. Sci. USA 97:13549-13554 (2000); Xu et al., Proc. Natl. Acad. Sci. USA 97:6379-6384 (2000); Xu

et al., Science 279:1922-1925 (1998)). While SRC-1 and RIZ1 deficient female animals show similar reduced sensitivity to female sex hormones, SRC-1 mutation, unlike RIZ1, also affects mammary duct growth during puberty in virgin mice and hormone response in males. The partial hormone resistance phenotypes observed in RIZ1 or SRC-1 deficient mice likely reflect overlapping functions of RIZ1 or SRC-1 related proteins, for which there are many. Or, it may be related to an additive effect of RIZ1 and SRC-1 on ER function. Reduced H3-K9 methylation, as induced by E2 inhibition of RIZ1, would, in and of itself, facilitate transcription activation by reducing the binding of the heterochromatin protein HP1 to methylated K9 on H3 (Bannister et al., Nature 410:120-124 (2001); Lachner et al., Nature 410:116-120 (2001)). In addition, transcription would be further enhanced by the acetylation of the methyl-free H3-K9 by SRC-1 or its related HATs.

The partial reduction in tissue response to steroids in RIZ1 deficient mice was accompanied by a corresponding reduction in target gene expression. E2 induction of progesterone receptor gene expression in the stroma and myometrium compartments of the uterus was reduced in RIZ1 deficient mice (Fig 2F-G). The result suggests a physiological role of RIZ1 in facilitating ER-transcriptional activation function and that such a coactivator function is likely responsible for the observed tissue response to E2.

While the overall tissue response to steroids is only partially reduced by RIZ1 deficiency, this does not necessarily indicate that RIZ1 is unable to serve a unique and non-redundant function in facilitating NHR function. It is possible that at the molecular level, RIZ1 may be uniquely involved in controlling expression



of certain target genes. Indeed, estrogen repressed progesterone receptor gene expression nearly 20-fold in the luminal epithelial cells of the uterus in the presence of RIZ1 but only 2-fold in its absence (Fig. 2F-G). Although ER/E2 appear to largely use RIZ1 as a coactivator, this result further suggests that it can also employ it as a corepressor in certain circumstances. It is possible that ER/E2 may enhance RIZ1 methylation of H3-K9 in certain cellular contexts.

That RIZ1 appears required for E2 induced tissue growth contrasts with its tumor suppressor role. To help understand the apparent paradox, the role of RIZ1 in breast cancer was studied. RIZ1 overexpression enhanced ER-dependent transactivation of reporter gene whereas overexpressing a RIZ1 antagonist, RIZ2, inhibited the transactivation function of endogenous ER in breast cancer cells. Thus, RIZ1 inactivation maybe associated with decreased hormone response in breast cancers. Consistently, RIZ1 silencing was shown to be significantly more common in E2 resistant or ER-negative comparing to ER-positive breast cancers. It is logical to expect that alteration of ER coactivators may play a role in the hormone response phenotypes of breast cancers. This idea has been pursued by several previous studies, which found overexpression of SRC3 (AIB1) and SRA in mostly ER-positive breast cancers (Anzick et al., Science 277:965-968 (1997); Kurebayashi et al., Clin. Cancer Res. 6:512-518 (2000); Murphy et al., Cancer Res. 60:6266-6271 (2000); Thenot et al., Mol. Cell. Endocrinol. 156:85-93 (1999)). As might be anticipated, there is a quantitative relationship between ER concentration in breast cancer tissue and response to E2 (Allegra, Semin. Oncol. 10:23-28 (1983); Osborne et al., Cancer 46:2884-2888 (1980)). The higher the ER amount, the more likely a response. ER is rarely mutated and the

term ER-negative refers to ER level that is below an empirically determined level. Partial hormone response still exists in such cells with low ER levels. It is therefore expected that loss in one of the coactivators  
5 (RIZ1) may further reduce hormone response of such cells. Similarly, silencing of a coactivator in ER-positive breast cancers, as shown here for RIZ1, may play a role in the acquisition of hormone resistance phenotypes of these tumors.

10 For hormone sensitive early stage breast cancer, the dual role of RIZ1 as a tumor suppressor and an ER-coactivator/growth promoter raises the issue whether disabling RIZ1 could be beneficial or not to tumor cell proliferation. It appears that retaining RIZ1  
15 may be more favorable than disabling it, in view of the reduction of E2-induced tissue growth in RIZ1 deficient mice. This view is also consistent with the finding that RIZ1 silencing was less common in ER-positive versus ER-negative breast cancers.

20 If RIZ1 may confer growth advantage to early stage breast cancers, why is it not so for late stage ER-negative breast cancers, where RIZ1 is commonly silenced? This is expected for a tumor suppressor that also has an ER-dependent growth promoter function. In  
25 breast cancers with functional ER, which, upon E2-binding, may turn RIZ1 from a default tumor suppressor into a coactivator/tumor promoter, RIZ1 is often present and may play a role in promoting tumor growth in the presence of E2. In these tumors, RIZ1 may also play a  
30 role in keeping tumor growth in check when E2 is absent. In contrast, in ER-negative breast cancers, where a coactivator would be no longer useful and a tumor suppressor would be undesirable, RIZ1 is frequently lost,

which may play a role in promoting the E2-independent growth phenotype of these tumors.

The questions arises as to why most other ER-coactivators are not silenced in hormone resistant breast cancers (Anzick et al., Science 277:965-968 (1997); Kurebayashi et al., Clin. Cancer Res. 6:512-518 (2000); Murphy et al., Cancer Res. 60:6266-6271 (2000); Thenot et al., Mol. Cell. Endocrinol. 156:85-93 (1999)). This is probably because they are not tumor suppressors. It is likely that only a coactivator with tumor suppressor function would represent a preferred target of inactivation during the transition from hormone sensitive to insensitive tumors. During such transition, hormone sensitive tumors will develop mechanisms to grow in the absence of hormonal stimuli or ER function. In these tumor cells that have survived compromised ER, a coactivator may not be useful anymore, and thus there will be no longer a selective pressure to keep it active. Meanwhile, in the absence of the ER switch, the default tumor suppressor function of a coactivator would be on, which would not be favorable to tumors and a selective pressure to inactivate the coactivator may emerge.

These results suggest that RIZ1 possesses intrinsic HMT activity. The RIZ1 specific region (1-200 aa) containing the PR-domain, which is absent in RIZ2, is sufficient for histone H4 methylation in vitro. The PR-domain (residues 30-160) is necessary but insufficient, and requires the less conserved regions (residues 161-200) C-terminal to the PR-domain for HMT activity. The requirement for the region nearby the PR-domain is similar to what has been described for the SET-domain (Rea et al., Nature 406:593-599 (2000)). The activity of the PR domain was impaired by tumor

associated point mutations, consistent with an important role of the activity in tumor suppression functions.

A new methyltransferase catalytic motif was identified, the PBD domain, located at the RIZ1 C-terminus. This domain can methylate H4 in vitro and can physically/functionally cooperate with the PR domain to produce higher HMT activity as well as to broaden substrate specificity. The PBD domain appears not to be significantly related to any proteins in the database. That this domain has HMT activity and can modulate PR activity is consistent with a potential role of this domain in tumor suppression, as indicated by its deletion owing to frameshift mutations in microsatellite unstable cancers.

These data show that a bacteria-expressed 200-residue peptide consisting of the PR-domain region of RIZ1 N-terminus methylated histone H4 while the full-length protein also used H1 and H3 as substrates. The result is consistent with a need for other regions of RIZ1, such as the PBD domain, in modulating the PR domain activity. Indeed, a novel H1 like-protein was methylated only in the presence of both PR and PBD but not by either alone. Alternatively, immunoprecipitated RIZ1 may not be free of RIZ1 bound proteins that could modulate its enzyme activity. RIZ1 may exist as part of a protein complex and the intact nature of such a complex may be required by HMT activity. An example of a HMT complex is the SUV39H1 complex of 650 kDa (Nishioka et al., Genes & Dev. 16:479-489 (2002)). Finally, mammalian specific post-translational modifications of RIZ1 could play a role in substrate specific methylation activities. The latter two possibilities are consistent with the substrate specific regulation of RIZ1 HMT activity by E2

and TPA, and should be an important topic for future investigations.

The results also show that lysine 9 of histone H3 is a major methylation target of RIZ1 protein. RIZ1 protein caused enhanced H3-K9 methylation in vivo and also methylated H3-K9 in vitro. H3-K9 methylation is linked with heterochromatin formation and gene silencing, which in turn, implicates a similar role for RIZ1. Histone H1 and H4 are also methylated by RIZ1 but the role of H1 and H4 methylations in gene transcription remains to be understood. A RIZ1 target residue was identified on H1 in vitro as lysine 25, which is consistent with the known methylation of H1-K25 in vivo (Ohe et al., J. Biochem. (Tokyo) 100:359-368 (1986)).

That E2 can maintain or enhance methylation of one substrate (H1) while decrease methylation of another (H3) suggests a complex mechanism of regulation of RIZ1 activity. The changes in H3 and H1 presumably together contribute to the ligand-dependent RIZ1 activation of ER transactivation functions. That H3-K9 methylation by RIZ1 is inhibited by E2 is consistent with RIZ1 as a coactivator, given that such methylation is linked with gene silencing. The contrasting change in H1 methylation suggests that the role of such methylation is likely to be at least different from, if not the opposite of, that of H3-K9 methylation. H1 phosphorylation is known to be important in transcriptional activation (Bhattacharjee et al., Mol. Cell. Biol. 21:5417-5425 (2001); Dou and Gorovsky, Mol. Cell. 6:225-231 (2000)), although the role of its methylation remains unknown. The finding here that RIZ1 or E2 induced both phosphorylation and methylation of H1 suggests a role for H1 methylation similar to that of H1 phosphorylation. In view of the coactivator function and the previously described

transcriptional repressor function (Xie et al., J. Biol. Chem. 272:26360-26366 (1997)), RIZ1 appears to have a dual role in both transcription activation and repression. The dual role may be mediated in part by

5 methylation of H3-K9 and H1-K25, respectively (Table 4), which may be turned more toward one direction or the other depending on the protein and promoter environment of RIZ1 at particular circumstances. When RIZ1 is recruited by estrogen to activate transcription, the H3

10 methylation function of RIZ1 would be inhibited and the H1 methylation would be maintained or enhanced. The results suggest a model that RIZ1 has a dual role of a tumor suppressor and an ER-coactivator/growth promoter and the same PR-domain is required for both. ER/E2

15 appears to be a switch that can turn RIZ1 from a tumor suppressor into a growth promoter. The dual role of RIZ1 may be mediated by different substrate usage. The H3-K9 methylation/gene repression function of RIZ1 appears to be associated with growth arrest whereas the H1

20 methylation is associated with cell growth, as indicated by the changes of these methylation activities of RIZ1 during terminal differentiation of U937 cells (summarized in Table 4). The tumor suppressor role of RIZ1 is non-tissue specific but the ER-coactivator function is

25 restricted to ER-positive tissues.

Table 4

RIZ1 function <sup>a</sup>	E2 target tissues or ER(+)breast cancer	Non-E2 target tissues or ER(-) breast cancer	Cell proliferation potential	
	+E2	- E2	high	low
5 H1-K25 methylation	++	+	++	+
H1 phosphorylation	+			
Gene activation				
Growth promotion				
10 H3-K9 methylation	+	++	+	++
Gene repression	++			
Growth suppression				

a The functional activity of RIZ1 is indicated as relatively low(+) or high(++)

It seems paradoxical that E2 employs a tumor suppressor for its growth promoting functions. That RIZ1 HMT activity is sensitive to E2 raises the possibility that RIZ1 may have a role as a sensor and breaker to prevent abnormal hormone-independent growth of cells. The presence of RIZ1 in early stage breast cancers may explain the lack of tumor growth in the absence of E2, while its common absence in advanced breast cancers may explain the hormone-independent tumor growth. E2 may serve as a switch to turn off the tumor suppressor function of RIZ1, perhaps by changing target genes and/or HMT activities. Given that the HMT function is required for both tumor suppressor and coactivator functions of RIZ1, E2 must not simply turn off all HMT activities. It is therefore reassuring that E2 can selectively inhibit

RIZ1 HMT activity toward one substrate (H3) while maintain or enhance the activity toward the other (H1).

In summary, the studies described above demonstrate that a HMT (RIZ1) is a target of estrogen and is required for efficient female sex hormone action in vivo. Compromised HMT function of RIZ1 is associated with estrogen resistant breast cancers, and may prove important in the transition to hormone resistance and hormone independent growth during breast cancer progression. By analogy, altered HMT function may also be involved in clinical syndromes characterized by an impaired female sex hormone homeostasis such as osteoporosis, cardiovascular disease, and Alzheimer's disease.

15

#### EXAMPLE II

This example shows various assays suitable for identifying RIZ1 modulatory compounds and compounds that modulate progesterone receptor activity.

A. *Biochemical assay based on binding of RIZ1 to universal inhibitors of protein methyltransferase activity.*

The best presently known universal inhibitor of PMT is S-Adenosyl-L- or D-homocysteine (SAH,  $K_i=5$  to  $12 \mu\text{M}$ ). A less potent inhibitor is Adenosyl-L-ethionine (25  $K_i=170 \mu\text{M}$ ). SAH mimics the structure of S-adenosyl-L-methionine (SAM) that is the substrate of PMT and the methylgroup donor. RIZ1 is expected to bind to SAH through its PR/PMT motif and its PBD domain.

Multi-well plates are coated with SAH and each well contacted with compounds from a small molecule



chemical compound library. The plates are incubated with purified recombinant protein containing the RIZ1 PR/PMT motif (about 200 amino acids) that has been labeled with a fluorescent probe. After washing away unbound RIZ1  
5 protein, the plates are read by a fluorescence detector.

*B. Biochemical assay based on binding of RIZ1 to ER or PR*

The coactivator function of RIZ1 on ER and PR is dependent upon physical complex formation. Binding of RIZ1 to ER and PR is mediated by the LXXLL motif of RIZ1  
10 and is hormone dependent.

Recombinant RIZ1 protein is coated onto multiwell plates and each well contacted with compounds from a small molecule chemical compound library, as well as estrogen or progesterone. The plates are incubated  
15 with purified recombinant ER or PR protein that is labeled with a fluorescent probe. After washing away unbound ER or PR protein, the plates are read by a fluorescence detector.

*C. Cellular assay based on RIZ1 coactivation of ER or PR*

20 Multiwell plates are seeded with CV1 cells and the cells transfected with a plasmid containing a ER responsive promoter linked to a luciferase reporter, a plasmid expressing ER, and a plasmid expressing RIZ1. Each well is contacted with compounds from a small  
25 molecule chemical compound library. Two days following transfection, the luciferase activity of each well is assayed and read by a fluorescence detector.

D. Assay to determine or verify the *in vivo* efficacy of modulators of RIZ1

Once target compounds are obtained through *in vitro* biochemical or cellular high throughput screening, the compounds are tested for *in vivo* efficacy in inhibiting/enhancing the physiological function of RIZ1. Female mice lacking RIZ1 PR/PMT function show decreased response to estrogen and progesterone. Therefore, inhibitors of RIZ1 PR/PMT are expected to decrease estrogen and progesterone responses in mice, while activators of RIZ1 may enhance responses.

Overectomized female mice are treated with estrogen and target compounds for three days. The animals are sacrificed and examined for uterine wet weight and vaginal cornification. In additon, mammary gland growth in response to estrogen and progesterone is determined. 8 week old female mice are ovariectomized at day 0 and treated with E2 (50 µg/day) + P4 (1 µg/day) from day 14-34 (or 21-day releasing hormone pellets containing 0.1 mg of E2 and 10 mg P4; Innovative Research of America). Target compounds are applied together with the hormones. On day 35, the mice are sacrificed and whole mounts prepared and stained. RIZ1 null mutant mice are similarly treated to serve as specificity control for the effects of the target compounds.

All journal article, reference and patent citations provided above, in parentheses or otherwise, whether previously stated or not, are incorporated herein by reference in their entirety.

Although the invention has been described with reference to the examples provided above, it should be understood that various modifications can be made without departing from the spirit of the invention.

What is claimed is:

1. A method of screening for a compound that modulates RIZ histone methyltransferase activity, comprising:
  - 5 a) contacting a RIZ or RIZ fragment having histone methyltransferase activity with one or more candidate compounds; and
  - b) determining histone methyltransferase activity of said contacted RIZ or RIZ fragment,
- 10 wherein a compound that modulates RIZ histone methyltransferase activity is identified.
2. The method of claim 1, wherein said RIZ is RIZ1.
3. The method of claim 1, wherein said RIZ is RIZ2.
4. The method of claim 1, wherein said RIZ fragment
- 15 comprises amino acids 1-200 of human RIZ1.
5. The method of claim 1, wherein said RIZ fragment comprises amino acids 1-332 of human RIZ1.
6. The method of claim 1, wherein said RIZ fragment comprises amino acids 1514-1680 of human RIZ1.
- 20 7. The method of claim 1, wherein histone H1 methyltransferase activity is determined.
8. The method of claim 1, wherein histone H3 methyltransferase activity is determined.
9. The method of claim 1, wherein histone H4
- 25 methyltransferase activity is determined.

10. A method of screening for a compound that modulates progesterone receptor activity, comprising:

- a) providing a RIZ1 modulatory compound;
  - b) determining the ability of said RIZ1 modulatory
- 5 compound to modulate progesterone receptor activity, wherein a compound that modulates progesterone receptor activity is identified.

11. The method of claim 10, wherein step a) comprises:

- 10 i) contacting RIZ1 with one or more candidate compounds;
  - ii) determining the ability of said candidate compound to selectively bind RIZ1; and
  - iii) providing a compound from step ii) that
- 15 selectively binds RIZ1.

12. The method of claim 10, wherein step a) comprises:

- i) contacting RIZ1 with one or more candidate compounds under conditions wherein RIZ1 selectively binds
- 20 a RIZ1-binding partner;
- ii) determining the ability of said candidate compound to modulate said selective binding between RIZ1 and said RIZ1-binding partner; and
  - iii) providing a compound from step ii) that
- 25 modulates said selective binding.

13. The method of claim 10, wherein step a) comprises:

- 5 i) contacting RIZ1 with one or more candidate compounds under conditions wherein RIZ1 exhibits a functional activity;
- ii) determining the ability of said candidate compound to modulate said functional activity; and
- iii) providing a compound from step ii) that modulates said functional activity.

10 14. The method of claim 13, wherein said functional activity is histone methyltransferase activity.

15 15. The method of claim 10, wherein step b) comprises determining the ability of said RIZ1 modulatory compound to modulate progesterone receptor-mediated transcription.

16. The method of claim 10, wherein step b) comprises determining the ability of said RIZ1 modulatory compound to modulate progesterone-dependent mammapoiesis.

20 17. The method of claim 10, wherein step b) comprises determining the ability of said RIZ1 modulatory compound to modulate progesterone-dependent uterine development.

25 18. A method of identifying an individual with an estrogen receptor positive (ER+) tumor having a reduced likelihood of responding to endocrine therapy, comprising determining the RIZ1 status of said tumor, wherein an abnormal RIZ1 status identifies said individual as an individual with a reduced likelihood of responding to endocrine therapy.

19. The method of claim 18, wherein abnormal RIZ1 status is evidenced by RIZ1 promoter methylation.

20. The method of claim 18, wherein abnormal RIZ1 status is evidenced by decreased RIZ1 protein expression.

5 21. The method of claim 18, wherein abnormal RIZ1 status is evidenced by decreased RIZ1 mRNA expression.

22. The method of claim 18, wherein abnormal RIZ1 status is evidenced by a mutation in the RIZ1 gene.

23. The method of claim 18, wherein abnormal RIZ1  
10 status is evidenced by altered RIZ1 histone methyltransferase activity.

24. The method of claim 23, wherein said altered RIZ1 histone methyltransferase activity is increased H1 methylation or decreased H3 methylation.

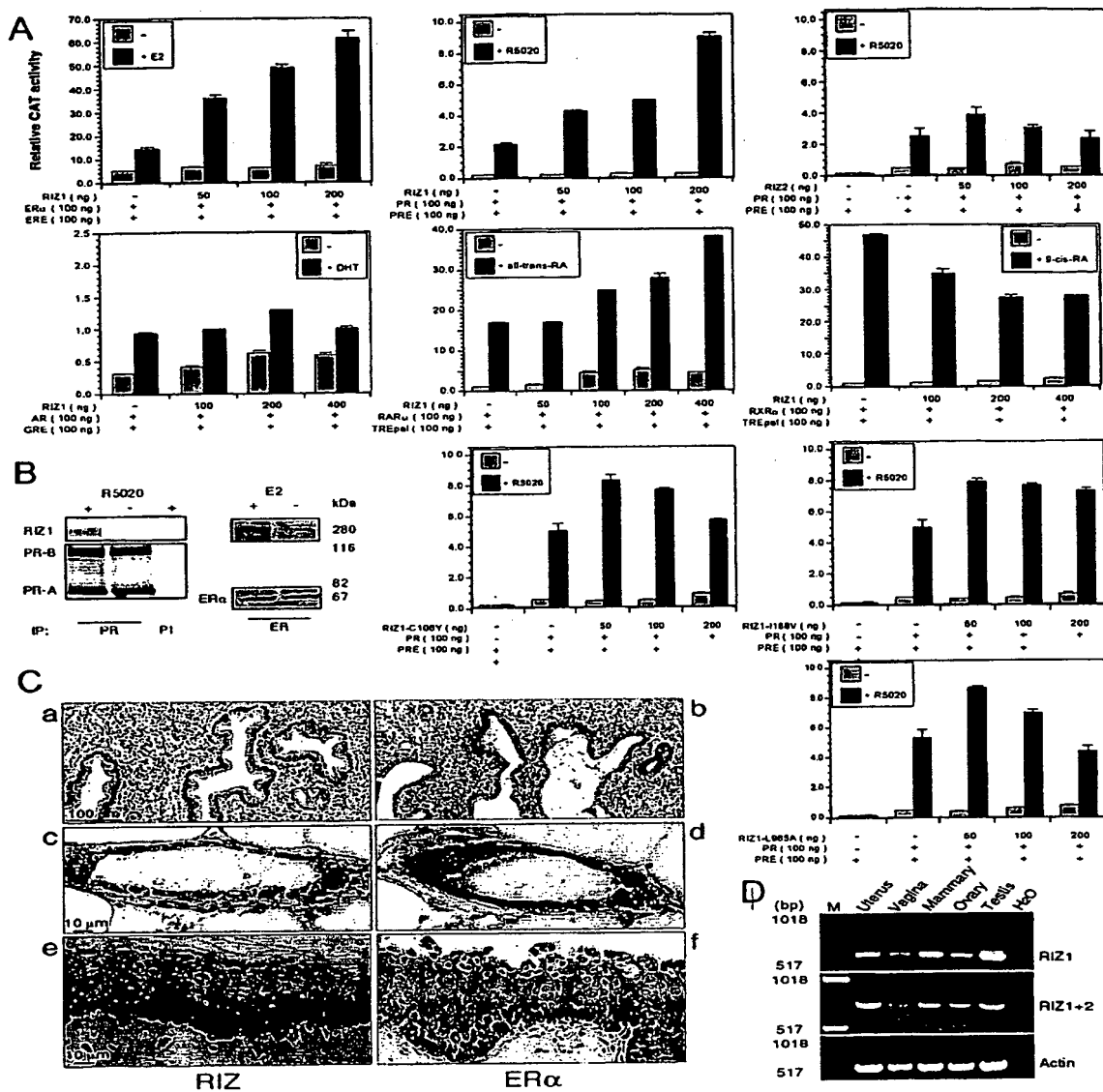


FIGURE 1



2/7

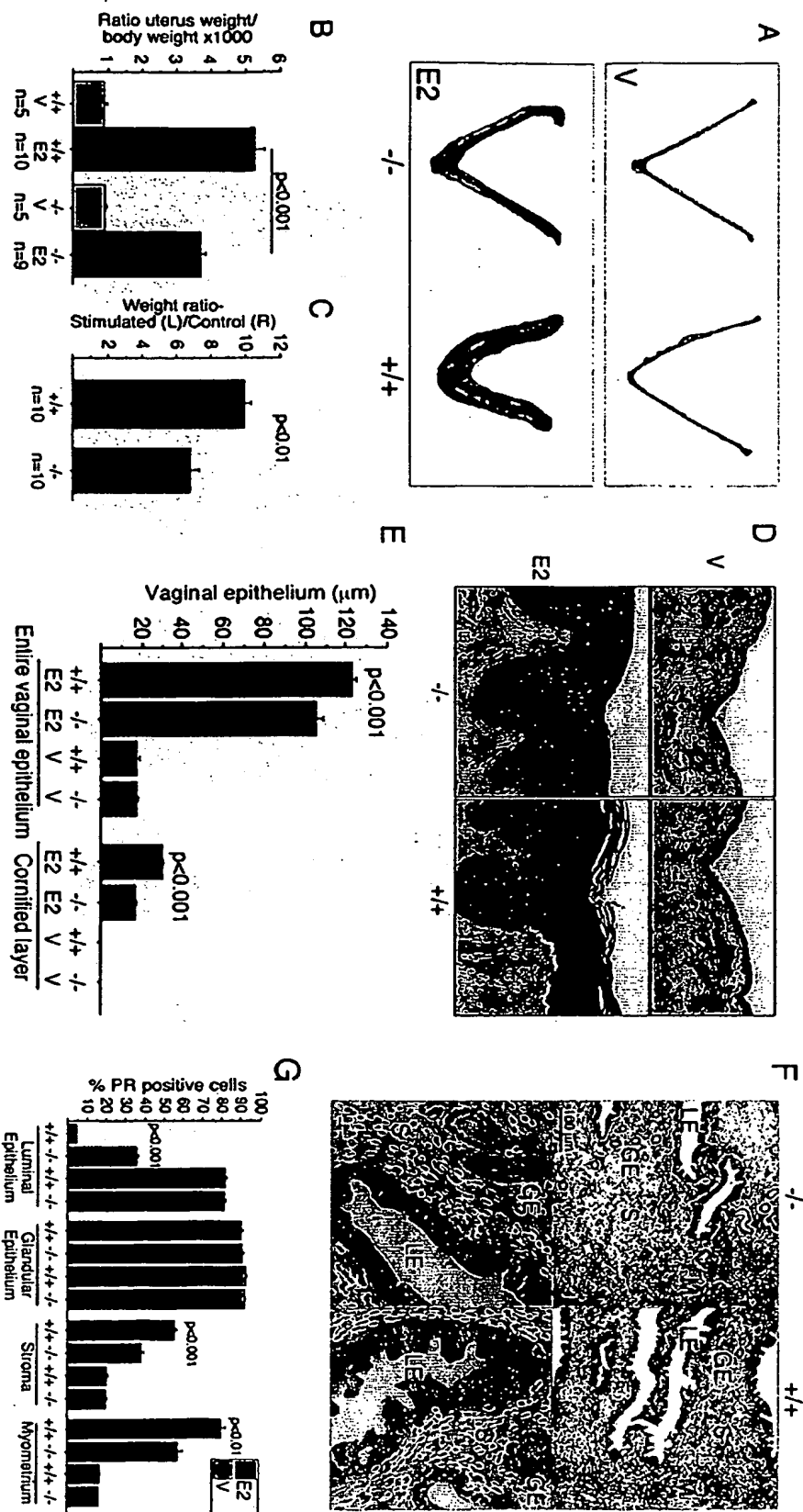


FIGURE 2

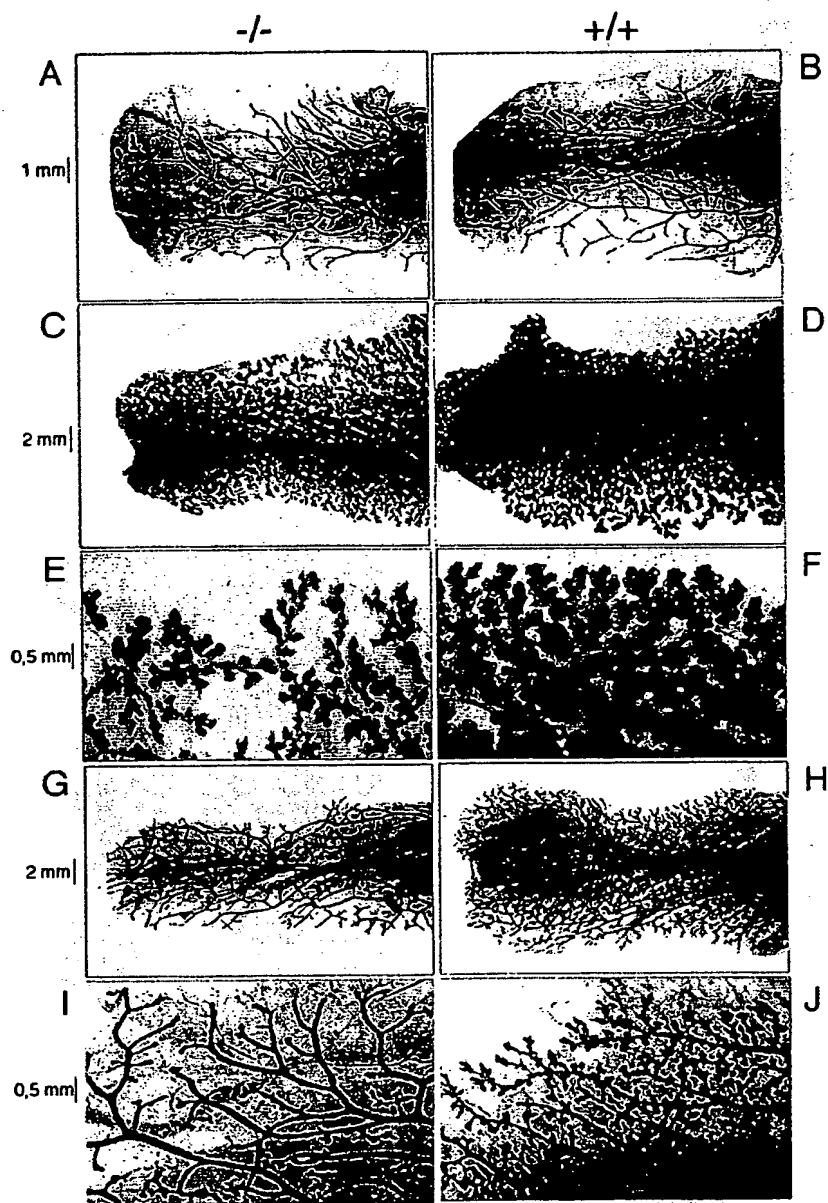


FIGURE 3

4/7

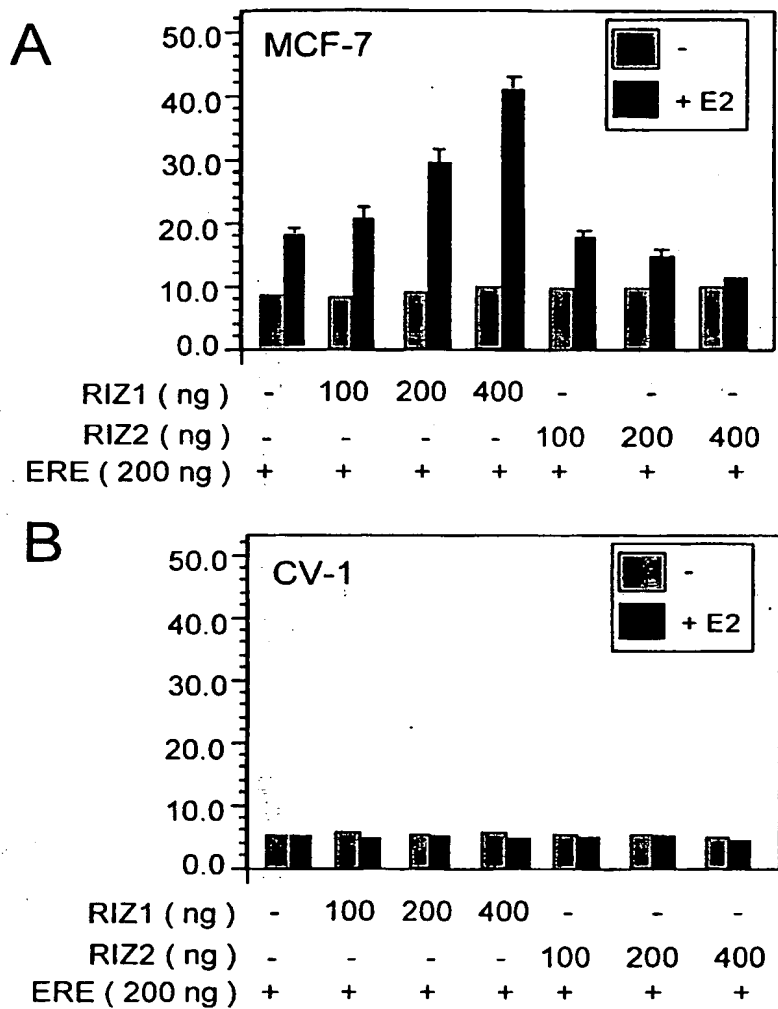


FIGURE 4

5/7

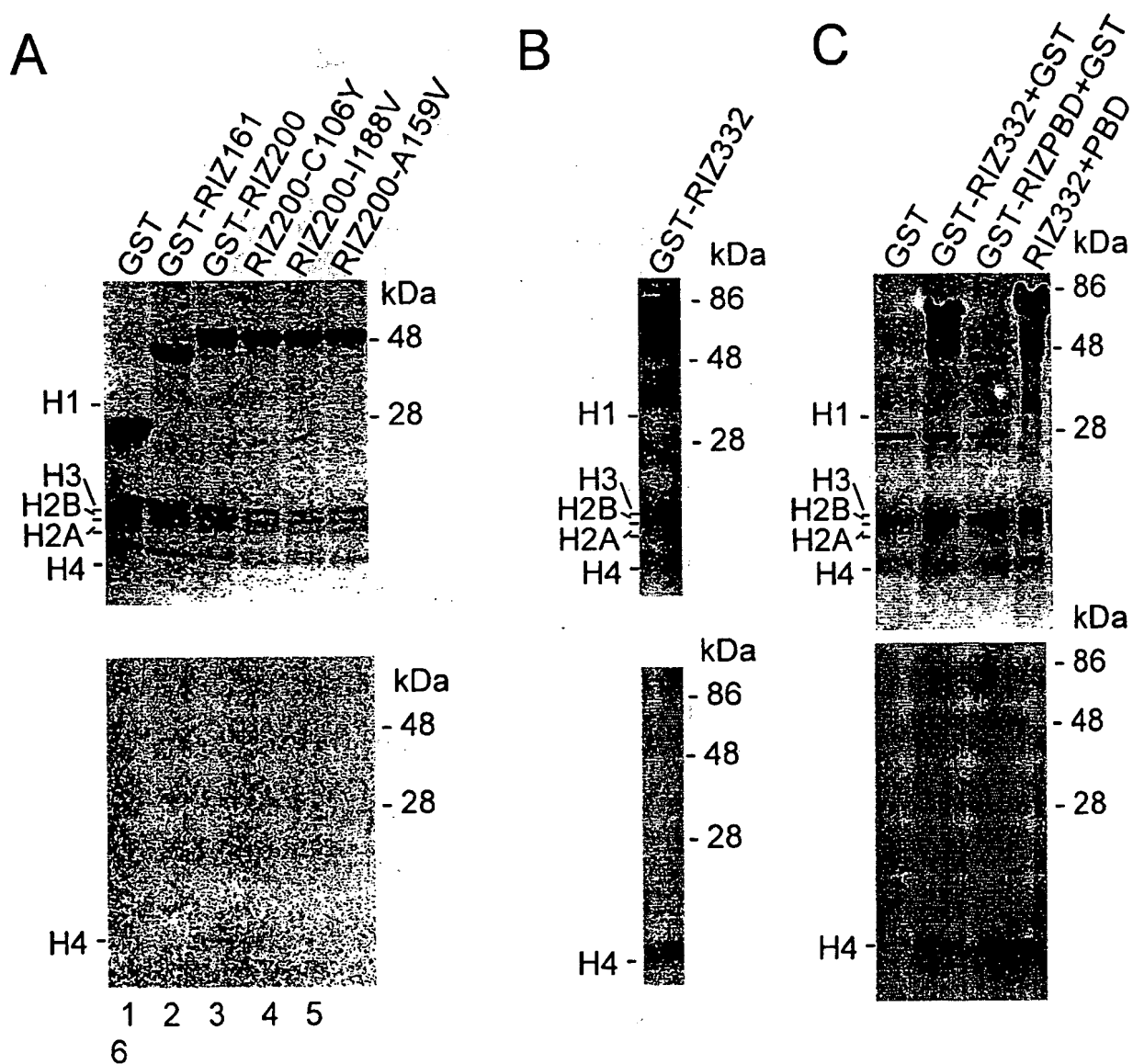


FIGURE 5

6/7

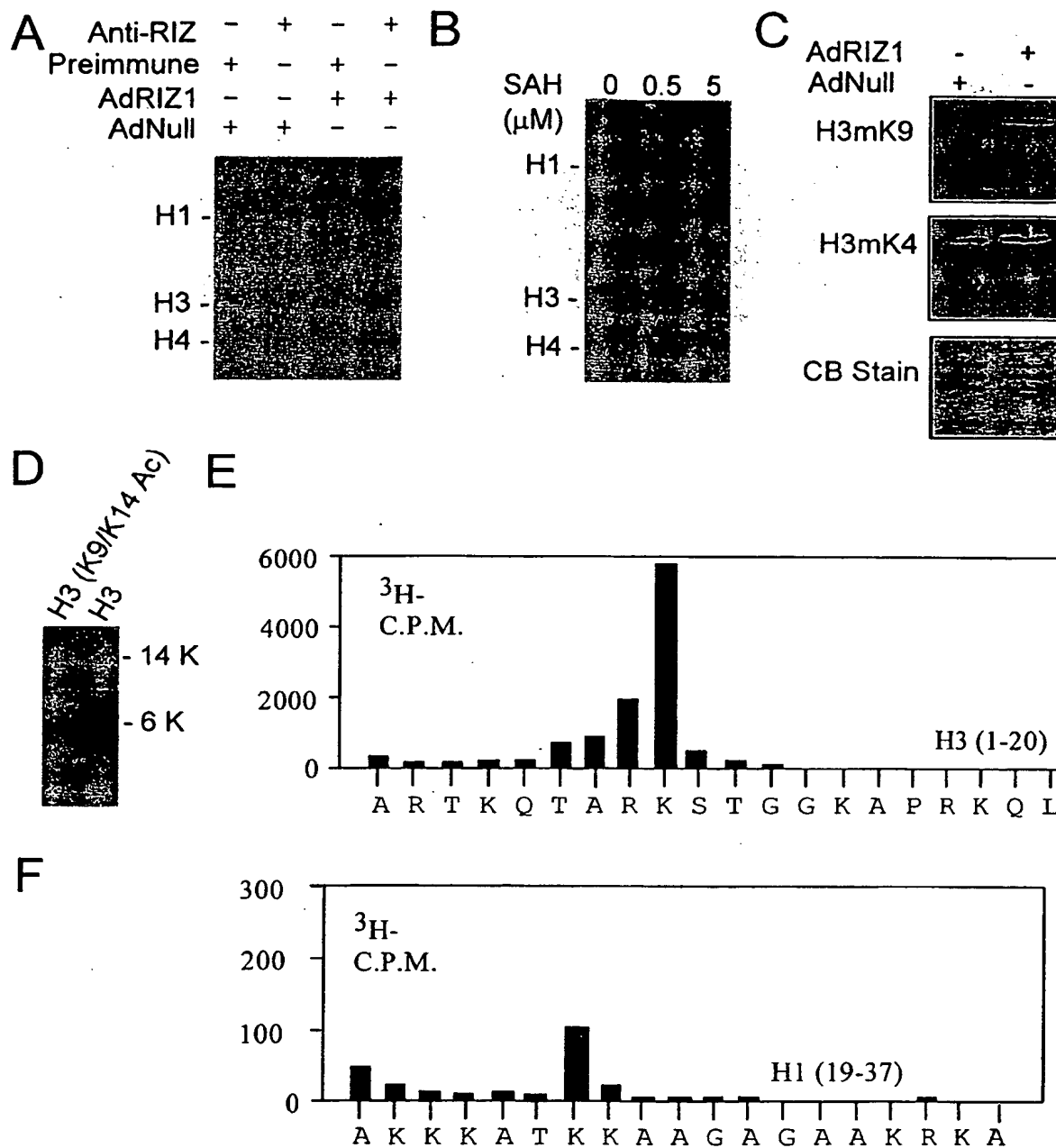


FIGURE 6

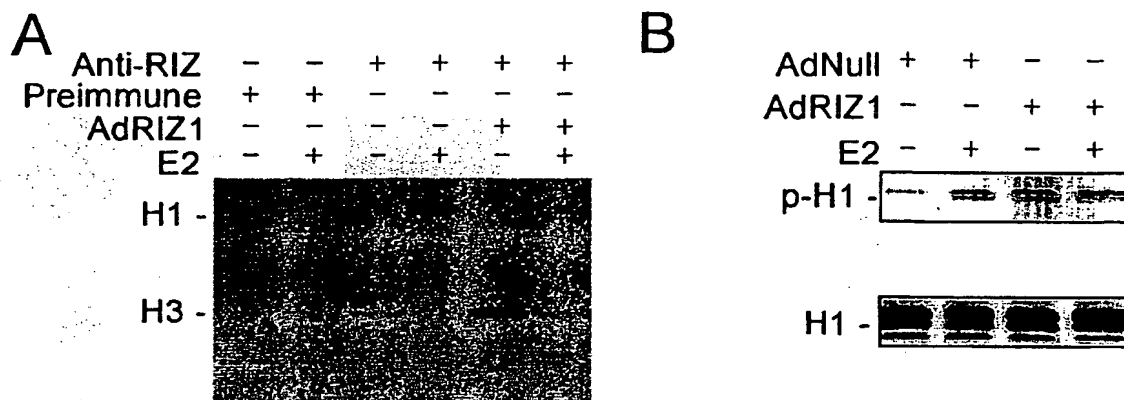


FIGURE 7

## SEQUENCE LISTING

<110> The Burnham Institute  
Huang, Shi

<120> Screening, Diagnostic and Therapeutic  
Methods Relating To RIZ

<130> FP-LJ 5225

<150> US 60/290,481

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- 15 -

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19

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